MOLECULAR CYTOGENETIC STUDIES ON RARE SOFT TISSUE SARCOMAS AND EWING TUMORS

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Academic dissertation

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To Mats, Jasper, and Rasmus

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1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

I Kiuru-Kuhlefelt S, El-Rifai W, Sarlomo-Rikala M, Knuutila S, Miettinen M: DNA copy number changes in alveolar soft part sarcoma: a comparative genomic hybridization study. Mod Pathol 11(3):227-31, 1998.

II Kiuru-Kuhlefelt S, Knuutila S, Miettinen M: Low-grade fibromyxoid sarcoma reveals recurrent DNA copy number losses at 13q21-q22 by comparative genomic hybridization. Submitted, 2001.

III Kiuru-Kuhlefelt S, Sarlomo-Rikala M, Larramendy ML, Söderlund M, Hedman K, Miettinen M, Knuutila S: *FGF4* and *INT2* oncogenes are amplified and expressed in Kaposi's sarcoma. Mod Pathol 13(4):433-7, 2000.

IV Kiuru-Kuhlefelt S, El-Rifai W, Fanburg-Smith J, Kere J, Miettinen M, Knuutila S: Concomitant DNA copy number amplification at 17q and 22q in dermatofibrosarcoma protuberans. Cytogenet Cell Genet 92 (3-4):192-5, 2001.

V Tarkkanen M, Kiuru-Kuhlefelt S, Blomqvist C, Armengol G, Böhling T, Ekfors T, Virolainen M, Lindholm P, Monge O, Picci P, Knuutila S, Elomaa I: Clinical correlations of genetic changes by comparative genomic hybridization in Ewing sarcoma and related tumors. Cancer Genet Cytogenet 114(1):35-41, 1999.

The publications are referred to by their Roman numerals in the text.

2. ABBREVIATIONS

ABL	Abelson oncogene
AIDS	acquired immunodeficiency syndrome
ASPL	alveolar soft part sarcoma chromosome region, candidate gene 1
ASPS	alveolar soft part sarcoma
ATF1	activating transcription factor 1 gene
ATM	ataxia telangiectasia mutated gene
bp	base pairs
BCL1	B-cell leukemia/lymphoma-1 gene
BCR	breakpoint cluster region gene
BRCA1, 2	breast cancer, early onset, genes 1 and 2
CCND1	cyclin D1 gene (alias: *PRAD1*)
CDK4	cyclin-dependent kinase 4 gene
CGH	comparative genomic hybridization
CML	chronic myeloid leukemia
COL1A1	collagen type I alpha 1 gene
DAPI	4',6-diamino-2-phenylindole
DDFS	distant disease-free survival
DDIT3	DNA-damage-inducible transcript 3 (alias: *CHOP10*)
DFSP	dermatofibrosarcoma protuberans
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
EMS1	mammary tumor and squamous cell carcinoma-associated gene
ERG	v-ets avian erythroblastosis virus E26 oncogene-related gene
ETS	Ewing's sarcoma translocation genes
ETV1, 4, 6	*ETS* variant genes 1, 4, and 6
EWS	Ewing's sarcoma breakpoint region 1 gene
FGF3	fibroblast growth factor 3 gene (alias: *INT2*)
FGF4	fibroblast growth factor 4 gene
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
FKHR	forkhead homolog 1 gene
FLG	filaggrin gene
FLI1	Friend leukemia virus integration 1 gene
FS-DFSP	fibrosarcomatously transformed dermatofibrosarcoma protuberans
FUS	translocated in liposarcoma gene
GAS	gastric cancer gene
GIST	gastrointestinal stromal tumor
GLI	glioma-associated oncogene homolog gene
HBV	hepatitis B virus
HCV	hepatitis C virus
HHV-8	human herpesvirus 8
HIV	human immunodeficiency virus
HSCT	hyalinizing spindle cell tumor with giant rosettes
HTLV-1	human T-cell leukemia virus type I
INT2	murine mammary tumor virus integration site (*v-int-2*) oncogene homolog gene (alias: *FGF3*)
KIT	*v-kit* Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog gene

KSHV	Kaposi's sarcoma-associated herpesvirus, HHV-8
LED	lupus erythematosus disseminatus
LGFMS	low-grade fibromyxoid sarcoma
LMS	leiomyosarcoma
Mb	megabase
MDM2	human homolog of murine double minute 2 gene
MFH	malignant fibrous histiocytoma
MMTV	mouse mammary tumor virus
MPNST	malignant peripheral nerve sheath tumor
NR4A3	nuclear receptor subfamily 4, group A, member 3 gene
NTRK1, 3	neurotrophic tyrosine kinase receptor genes, types 1 and 3
PAT1	amyloid beta precursor protein-binding protein 2 gene
PAX 3, *7*	paired box homeotic genes 3 and 7
PDGFB	platelet-derived growth factor beta polypeptide gene
PRAD1	parathyroid adenomatosis 1 gene (alias: *CCND1*)
PS6K	ribosomal protein polypeptide gene
RAD51C	homolog C of S. cerevisiae *RAD51* gene
RB1	retinoblastoma gene
SAS	sarcoma amplified sequence gene
SIGMA1B	adaptor-related protein complex 1, sigma 2 subunit gene
SPRR3	small proline-rich protein 3 gene
SSX1, *2*, 4	synovial sarcoma, X breakpoint genes 1, 2, and 4
SYT	synovial sarcoma translocation gene
TAF2N	TATA box binding protein-associated factor 2N gene
TBX2	T-box 2 gene
TFE3	transcription factor for immunoglobulin heavy-chain enhancer 3 gene
TP53	tumor protein 53 gene
TRITC	tetra-rhodamine isothiocyanate
WT1	Wilms tumor 1 gene
YAC	yeast artificial chromosome

3. ABSTRACT

The identification of genetic abnormalities in many human tumor types has led to tremendous growth in our understanding of tumorigenesis. This thesis contributes to research on soft tissue sarcomas, particularly rare types, by its use of novel molecular cytogenetic methods, which make it possible to study archival samples and to analyze even the very complex genetic aberrations commonly seen in soft tissue sarcomas. Genetic alterations in 13 alveolar soft part sarcomas (ASPS), 11 low-grade fibromyxoid sarcomas (LGFMS), 12 Kaposi's sarcomas, 19 dermatofibrosarcoma protuberances (DFSP), and 28 Ewing tumors were studied by the recently developed comparative genomic hybridization method. The Kaposi's sarcoma tumors were further studied by interphase fluorescence *in situ* hybridization (FISH) and immunohistochemistry.

No recurrent specific DNA copy number alterations were found in the ASPS tumors. In one of the tumors, gain of Xp11-pter was recognized, probably reflecting the presence of the recently discovered specific unbalanced translocation der(17)t(X;17)(p11;q25).

Of the 11 LGFMS tumors, six presented with a DNA copy number loss of 13q21-q22. This exceptionally high incidence (55%) may suggest an important role for a tumor suppressor gene(s), located at 13q21-q22, in this particular tumor type.

A recurrent, restricted gain of 11q13, was identified as being present in four Kaposi's sarcomas. This area harbors several known oncogenes, of which two, *FGF4* and *INT2*, were considered to be of special interest in this context. The amplification of *FGF4* and *INT2* was therefore further studied by interphase FISH with YAC probes carrying both genes. The genes were found to be amplified. The expression of these genes was studied by immunohistochemistry, which revealed immunoreactivity with both anti-FGF4 and anti-INT2. These experiments showed that both *FGF4* and *INT2* were amplified and expressed in Kaposi's sarcoma, possibly contributing to the pathogenesis.

Gains or amplifications of 17q21-qter and 22pter-q13 were found in 100% and 79% of the DFSP tumors, respectively. The starting points of the gains or amplifications coincided in many cases with the breakpoints of the DFSP-specific translocation t(17;22)(q22;q13). Although the translocation, often located in supernumerary ring chromosomes creating amplification of the chromosomal segments involved, is

considered to be the tumorigenetic event, the consistency of the gains may imply an important primary role in DFSP pathogenesis for genes located at 17q21-qter and 22pter-q13.

The possible role of DNA copy number changes in DFSP tumor progression was evaluated by comparing the DNA copy number alterations of typical DFSP tumors to the alterations of DFSP tumors with areas of fibrosarcomatous transformation, which is considered to represent tumor progression. No significant differences were noted between the two groups, suggesting mechanisms other than copy number alterations to be more important in DFSP progression.

The most recurrent copy number alterations found in the Ewing tumors were statistically tested for possible correlations with clinical parameters. DNA copy number increases of 1q21-q22, of chromosome 8, and of chromosome 12 were associated with trends towards worse prognosis, but no statistically significant correlations were reached. Gain or high-level amplification of 6p21.1-pter correlated significantly with worse prognosis. Because the patients with this aberration had, however, clinical signs with poor prognosis, the independent relevance of 6p21.1-pter gain as a prognostic marker remains to be confirmed.

Characterization of genomic areas comprising DNA copy number losses or gains will potentially affect the biological and clinical assessment of soft tissue sarcomas, and novel findings of genetic changes can allow the design of molecularly targeted treatments.

4. INTRODUCTION

All malignant neoplasms are known to be caused by genetic aberrations. These aberrations include deletions, translocations, inversions, and amplifications which disrupt normal functions of cellular oncogenes, tumor suppressor genes, mismatch repair genes, and other genes like those involved in cell apoptosis. In most cancer cases, accumulation of various alterations in the cellular DNA is needed for malignant transformation (Vogelstein & Kinzler, 1993). Many malignant diseases have already been genetically well characterized, and cytogenetic as well as molecular genetic methods have been successfully applied in their diagnostic assessment. This is particularly true for hematological malignancies, the clinical evaluation of which today routinely includes genetic analysis (Clare & Hansen, 1994).

Soft tissue sarcomas are a heterogeneous group of malignant connective tissue tumors of mesodermal or neuroectodermal origin. The spectrum of tumors in this entity is broad, which makes differential diagnosis and tumor classification a challenging task. Cytogenetically, soft tissue sarcomas frequently carry complex aberrations; this poses difficulties for analyzing the tumors by conventional cytogenetic approaches (Sreekantaiah *et al*., 1994).

Since especially the rarer soft tissue sarcomas frequently present noticeable diagnostic problems, and even many of the better-characterized tumors still lack reliable prognostic markers, new specific molecular genetic markers are expected to become increasingly useful in the clinical evaluation of such tumors. In addition to the diagnostic and prognostic impact, identification of new genetic events in the disease process will increase our understanding of the biology of cancer development and progression. This will ultimately reveal new therapeutic targets, as has already eventuated in treatment for chronic myeloid leukemia (Druker *et al*., 2001b). This research aims at increasing the understanding of genetic alterations involved in the pathogenesis of rare soft tissue sarcomas, and contributes to providing new prospects for their biological as well as clinical perception.

5. REVIEW OF THE LITERATURE

5.1. GENERAL CHARACTERISTICS OF SOFT TISSUE SARCOMAS

Soft tissue sarcomas can be defined as malignant tumors arising from nonepithelial extraskeletal tissues of the body, exclusive of the reticuloendothelial system, glia, and supporting tissue of parenchymal organs. Embryologically, soft tissue sarcomas are principally derived from the mesoderm, with some contribution from the neuroectoderm. They comprise malignant neoplasms of voluntary muscles, fat, fibrous tissue, and the vessels serving these tissues (Enzinger & Weiss, 1995).

Soft tissue sarcomas are clinically and histologically a highly heterogeneous group of tumors, some exhibiting only local aggressivity, others showing extensive metastatic potential. Their classification has earlier predominantly been based on the nuclear configuration of the tumor cells, thus providing a mainly descriptive classification. Currently, the more informative classifications used are based principally on the line of differentiation of the tumor, that is, the resemblance of the tumor tissue to normal adult tissue (Weiss, 1994). For clinical purposes, additional information is provided by staging and grading, which allow more accurate definition of the prognosis and planning of therapy. Other factors affecting prognosis include age and sex of the patient, as well as location of the tumor (Levine, 1999). The overall 5-year survival rates vary from less than 50% to over 80% depending on these factors (Lawrence *et al*., 1987; Pisters & Pollock, 1999; Russell *et al*., 1977; Rydholm *et al*., 1999).

Soft tissue sarcomas are relatively uncommon. Annually, the incidence rate is about one to two cases per 100 000 population (Hashimoto, 1995). In Finland around 130 new cases are diagnosed every year, and of all new cancer cases, less than 1% represent soft tissue sarcomas. The most common histological soft tissue sarcoma types - malignant fibrous histiocytoma (MFH), liposarcoma, leiomyosarcoma (LMS), and synovial sarcoma - comprise the majority of all cases among the over 50 histological entities recognized (Figure 1) (Bauer *et al*., 1999; Hashimoto, 1995; Kransdorf, 1995). The apparent difficulties in diagnostics and classification are illustrated by the substantial proportion of unclassified cases; as many as 5 to 10% of all soft tissue sarcomas fall into this category. Soft tissue sarcomas occur more commonly in males, and in the middle-aged and elderly, but distinct histological types show different sex and

age distributions. For example, MFH is typically a tumor of middle and old age, whereas rhabdomyosarcoma occurs almost exclusively among young patients. The anatomic location most often involved is the lower extremities, the thigh being the most frequent site, followed by the retroperitoneum, mesentery, and trunk (Bauer *et al*., 1999; Hashimoto, 1995; Kransdorf, 1995).

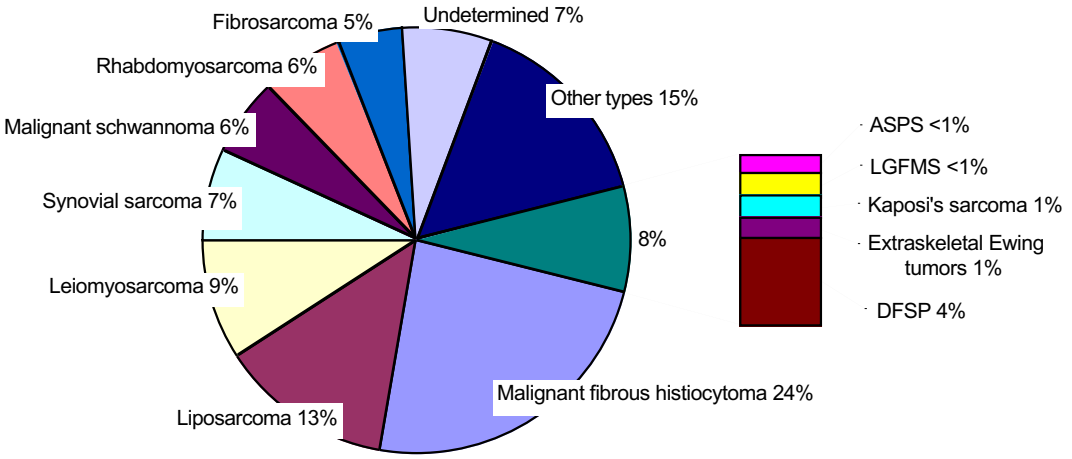

Figure 1. Mean frequency distribution of soft tissue sarcoma types according to Hashimoto (Hashimoto, 1995) and Kransdorf (Kransdorf, 1995). Incidences vary among the series; in the Scandinavian Sarcoma Group Register, MFH accounts for approximately 40% of all soft tissue sarcomas (Bauer *et al*., 1999).

The etiology or pathogenesis of soft tissue sarcomas, as of other types of malignant neoplasms, is still unknown. Benign soft tissue tumors rarely evolve into sarcomas, except for plexiform neurofibromas of patients with type 1 neurofibromatosis, which sometimes give rise to malignant schwannomas (malignant peripheral nerve sheath tumors, MPNST) (King *et al*., 2000). Evaluation of the causal factors in soft tissue sarcoma pathogenesis is difficult, since the period between the potential exposure and detection of the tumor is long, and various environmental and hereditary variables may effect simultaneously. Risk factors like radiation, viral infections, immunodeficiency, and dioxin exposure have, however, been recognized.

Ionizing radiation can induce many types of malignancies, including soft tissue sarcomas, by generating DNA damage. The most common post-radiation soft tissue

sarcoma is MFH, accounting for the majority of radiation-induced soft tissue sarcomas. Post-radiation sarcomas, although rare, appear to be associated with poorer prognosis than their sporadic counterparts (Davidson *et al*., 1986; Enzinger & Weiss, 1995; Laskin *et al*., 1988; Robinson *et al*., 1988; Wiklund *et al*., 1991).

Viruses are associated with a variety of human malignancies: hepatitis B virus (HBV) and hepatitis C virus (HCV) with hepatocellular carcinoma; Epstein-Barr virus (EBV) with Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, and childhood LMS; and human T-cell leukemia virus type I (HTLV-1) with adult T-cell leukemia. In addition, several candidate tumor viruses have been suggested, including human herpesvirus 8 (HHV-8), the role of which in the pathogenesis of Kaposi's sarcoma is currently under intensive research (Beral & Newton, 1998; Boman *et al*., 1997; Butel, 2000; Hill *et al*., 1997; Rogatsch *et al*., 2000; Schulz & Moore, 1999).

Dioxin exposure belongs among the environmental factors implicated in soft tissue sarcoma etiology, but the exposure time and level as well as the latent period are still equivocal (Bertazzi *et al*., 1997; Fingerhut *et al*., 1984; Saracci *et al*., 1991).

The genetic factors causing hereditary susceptibility for soft tissue sarcomas are discussed in a subsequent chapter.

5.2. GENERAL CHARACTERISTICS OF SARCOMAS STUDIED IN THIS WORK

Alveolar soft part sarcoma

Alveolar soft part sarcoma (ASPS) was first described as a distinct entity in 1952 (Christopherson *et al*., 1952). It is a rare tumor making up less than 1% of all soft tissue sarcomas. ASPS is typically a tumor of children and young adults, with female preponderance, occurring in a variety of anatomic locations (Hashimoto, 1995; Lawrence *et al*., 1987). The cellular nature of ASPS is poorly known. Several theories have existed as to the nature of ASPS, of which skeletal muscle differentiation has received the most convincing evidence (Auerbach & Brooks, 1987; DeSchryver-Kecskemeti *et al*., 1982; Miettinen & Ekfors, 1990b; Mukai *et al*., 1986; Nakano *et al*., 2000). Although the clinical course of ASPS is slow, eventually most of the patients develop metastases. The approximate median survival rate ranges from 3 to 11 years, depending on whether metastasis is present at diagnosis (Lieberman *et al*., 1989).

Low-grade fibromyxoid sarcoma

Low-grade fibromyxoid sarcoma (LGFMS) was described by Evans in 1987. Ten years later, a closely similar tumor, the hyalinizing spindle cell tumor with giant rosettes (HSCT), was characterized and later suggested to represent the same entity as LGFMS (Folpe *et al*., 2000; Lane *et al*., 1997). Because fewer than 100 cases of LGFMS have been reported, its incidence thus far remains unknown. LGFMS is a benign-appearing tumor typically occurring in deep soft tissues of young adults, but is reported also in children. This tumor was initially described as aggressive, and the death rate was suggested to be close to 20% (Devaney *et al*., 1990; Evans, 1993; Goodlad *et al*., 1995). Recently, however, more favorable figures have been proposed for metastasis and tumor-related deaths, possibly due to better recognition of LGFMS in the initial diagnostic evaluation of these patients. In addition to better diagnostics, classification of this tumor type will also affect its prognosis, since HSCT is rarely a metastatic tumor (Folpe *et al*., 2000; Lane *et al*., 1997).

Kaposi's sarcoma

Kaposi's sarcoma is a vascular tumor presenting in different clinico-pathological and epidemiological forms. The classical Kaposi's sarcoma has a prolonged, indolent course, affects mainly the extremities of elderly men, and is prevalent in certain parts of the world where it is endemic: the Mediterranean area and certain parts of Africa. In Central Africa, Kaposi's sarcoma may have accounted for up to 9% of all reported malignant neoplasias even before the acquired immunodeficiency syndrome (AIDS) epidemic (Safai & Good, 1981). The lymphadenopathic form of Kaposi's sarcoma is more rare, occurring primarily in young African children, with a less evident male preponderance. This form engages primarily the lymph nodes and has an aggressive clinical course (Desmond-Hellmann & Katongole-Mbidde, 1991). The immunosuppression-associated form of Kaposi's sarcoma occurs in adults and children undergoing immunosuppressive treatment, usually in a post-transplant situation. The course of the disease is dependent on immunosuppression level, and the lesions may regress after discontinuation of therapy. The incidence of post-transplantation Kaposi's sarcoma in Western countries has been estimated at less than

1% (Harwood *et al*., 1979; Qunibi *et al*., 1993). The AIDS-related form is the most prevalent, in the USA affecting up to 15 to 20% of 30- to 38-year-old homosexual or bisexual men with AIDS. The risk in other human immunodeficiency virus (HIV) transmission groups, such as hemophiliacs and drug addicts, has been less than 5%. Kaposi's sarcoma is the most common AIDS-related neoplasm, often manifesting as a disseminated, rapidly progressing disease in young adults (Beral, 1991; Desmond-Hellmann & Katongole-Mbidde, 1991).

Its epidemiological characteristics have suggested an infectious factor important in Kaposi's sarcoma etiology. Recent studies have strengthened this hypothesis, since a novel herpesvirus, HHV-8 or Kaposi's sarcoma-associated herpesvirus (KSHV), was identified and found to be present in all forms of Kaposi's sarcoma (Chang *et al*., 1994; Martin *et al*., 1998; Warmuth & Moore, 1997; Whitby *et al*., 1995). HHV-8 encodes several homologs of human genes with contribution to cellular signaling and regulatory pathways, but the exact roles of these genes in the transformation process remain to be defined (Boshoff & Chang, 2001).

Dermatofibrosarcoma protuberans

Dermatofibrosarcoma protuberans (DFSP) accounts for a few percent of all soft tissue sarcoma cases (Hashimoto, 1995). DFSP is a slow-growing, low-grade malignancy with a tendency for local recurrence, but a low potential for metastasis. It is typically a tumor of young to middle-aged adults, seen mainly on the trunk and the proximal extremities (Taylor & Helwig, 1962). A subset of DFSP tumors shows areas with fibrosarcomatous transformation (FS-DFSP), transformation considered a form of tumor progression associated with a significantly more aggressive clinical course: only 1% of the typical DFSP tumors metastasize, whereas in FS-DFSP cases metastases and tumor-related deaths have been reported in more than 10% and 5% (Mentzel *et al*., 1998).

Ewing tumors

Ewing's sarcoma and the closely related peripheral primitive neuroectodermal tumors, collectively often referred to as the Ewing tumors, can arise in bone or soft tissue. These tumors are typically found in children, adolescents, and young adults, with a median age

of 20 years, and account for approximately 14% of bone sarcomas and 2 to 3% of malignant soft tissue tumors (Bauer *et al*., 1999; Harms, 1995; Hashimoto, 1995). Ewing's sarcoma is a highly malignant disease, but with modern therapy its prognosis has improved, with 5-year survival rates over 50% reported(Elomaa *et al*., 2000; Schmidt *et al*., 1991).

5.3. GENETIC ABERRATIONS INVOLVED IN SOFT TISSUE SARCOMA AND EWING TUMORS

Cytogenetic changes

Cytogenetic studies have revealed chromosomal aberrations in practically all soft tissue sarcoma types. The aberrations include primary, tumor-specific alterations, which can be considered to reflect underlying gene defects, which have an important role in the tumorigenesis. Secondary changes are less specific, and often play an important role in tumor progression. More random, often multiple different changes, also appear, reflecting the genetic instability of the tumor (Mitelman, 1996; Nilbert, 1997).

Cytogenetically, several types of soft tissue sarcomas are characterized by specific chromosomal translocations, which, in addition to the sarcomas, are typical and often well characterized in hematological malignancies, but appear uncommon in carcinomas (Åman, 1999). Chronic myeloid leukemia (CML) is a classic example of a hematological malignancy with a specific translocation: The t(9;22), cytogenetically seen as the Philadelphia (Ph) chromosome, forms a fusion gene, *BCR-ABL*, encoding a protein with deregulated tyrosine kinase activity. Recently, very impressive treatment results have been achieved with a synthetic inhibitor of the activated tyrosine kinase (Druker *et al*., 2001a; Druker *et al*., 2001b). This inhibitor, known as STI571, appears to be extremely efficient and well tolerated. This represents the first example of a therapy specifically targeted to the malignancy-causing gene defect.

In addition to hematological malignancies, many of the specific translocations in soft tissue sarcomas have also been further described at molecular level (Table 1). In general, these translocations join together segments of two genes, forming novel fusion genes which often give rise to aberrant transcription factors (Fletcher, 1994). Specific translocations have been shown to be of clinical value in several instances in evaluation

of soft tissue sarcomas. In liposarcoma, the distinction between different histological subtypes, as well as differential diagnosis between liposarcoma and other sarcomas, can be facilitated by the presence of the characteristic translocations t(12;16)(q13;p11) and t(12;22)(q13;q12) in the myxoid and round cell subtypes of liposarcoma (Panagopoulos *et al*., 1996; Turc-Carel *et al*., 1986).

Table 1. Specific translocations in sarcomas

Tumor	Translocation	Affected genes[1]	References
Alveolar soft part sarcoma	t(X;17)(p11;q25)	*TFE3*/*ASPL*	(Ladanyi *et al*., 2001)
Congenital fibrosarcoma	t(12;15)(p13;q25)	*ETV6*/*NTRK3*	(Knezevich *et al*., 1998)
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	*WT1*/*EWS*	(Ladanyi & Gerald, 1994)
Dermatofibrosarcoma protuberans	t(17;22)(q22;q13)	*COL1A1*/*PDGFB*	(Simon *et al*., 1997)
Ewing tumors (Ewing's sarcoma, Askin tumor, and peripheral primitive neuroectodermal tumors)	t(11;22)(q24;q12) t(21;22)(q22;q12) t(7;22)(p22;q12)[2] t(17;22)(q21;q12)[2] t(2;22)(q33;q12)[2]	*FLI1*/*EWS* *ERG*/*EWS* *ETV1*/*EWS* *ETV4*/*EWS* *FEV*/*EWS*	(Delattre *et al*., 1992) (Zucman *et al*., 1993b) (Jeon *et al*., 1995) (Kaneko *et al*., 1996) (Peter *et al*., 1997)
Extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12) t(9;17)(q22;q11)	*NR4A3*/*EWS* *NR4A3*/*TAF2N*	(Labelle *et al*., 1995) (Panagopoulos *et al*., 1999)
Liposarcoma myxoid and round cell	t(12;16)(q13;p11)	*DDIT3*/*FUS*	(Rabbitts *et al*., 1993) (Knight *et al*., 1995)
Rhabdomyosarcoma, alveolar	t(2;13)(q35;q14) t(1;13)(p36;q14)	*PAX3*/*FKHR* *PAX7*/*FKHR*	(Galili *et al*., 1993) (Davis *et al*., 1994)
Synovial sarcoma	t(X;18)(p11.2;q11.2)	*SSX1*/*SYT* *SSX2*/*SYT* *SSX4*/*SYT*	(Clark *et al*., 1994) (Crew *et al*., 1995) (Skytting *et al*., 1999a)
Soft tissue clear cell sarcoma (Malignant melanoma of the soft parts)	t(12;22)(q13;q12)	*ATF1*/*EWS*	(Zucman *et al*., 1993a)

[1] abbreviations for genes: *TFE3,* transcription factor for immunoglobulin heavy-chain enhancer 3 gene; *ASPL,* alveolar soft part sarcoma chromosome region, candidate 1; *ETV1, 4,* and *6, ETS* variant genes 1, 4, and 6; *NTRK3*, neutrotrophic tyrosine kinase receptor type 3 gene; *WT1*, Wilms tumor 1 gene; *EWS*, Ewing's sarcoma breakpoint region 1 gene; *COL1A1*, collagen type I alpha 1 gene; *PDGFB*, platelet-derived growth factor beta polypeptide gene; *FLI1*, Friend leukemia virus integration 1 gene; *ERG*, v-ets avian erythroblastosis virus E26 oncogene related gene; *NR4A3*, nuclear receptor subfamily 4, group A, member 3 gene; *TAF2N*, TATA box binding protein-associated factor 2N gene; *DDIT3*, DNA-damage-inducible transcript 3 gene; *FUS*, translocated in liposarcoma; *PAX 3* and *7*, paired box homeotic genes 3 and 7; *FKHR*, forkhead homolog 1 gene; *SSX1*, *2*, and 4, synovial sarcoma, X breakpoint genes 1, 2, and 4; *SYT*, synovial sarcoma translocation gene; *ATF1*, activating transcription factor 1 gene

[2] described in Ewing's sarcoma but not in other tumors of the Ewing tumor group

In synovial sarcoma, most tumors present with t(X;18)(p11.2;q11.2), which, at the molecular level, has been found to include three alternative gene rearrangements: The *SYT* gene at 18q11.2 is fused with either the *SSX1*, *SSX2*, or *SSX4* gene at Xp11.2. Identification of different fusion genes in synovial sarcoma has been suggested to be of prognostic value, but the specificity of the translocation was recently questioned by O'Sullivan et al., who found the *SYT-SSX* fusion in a substantial number of MPNSTs (Clark *et al*., 1994; de Leeuw *et al*., 1995; Inagaki *et al*., 2000; Nilsson *et al*., 1999; O'Sullivan *et al*., 2000; Skytting, 2000; Skytting *et al*., 1999a).

The Ewing tumors are characterized by translocations that fuse the *EWS* gene on chromosome 22q12 with one of the members of the *ETS* family of transcription factors: *FLI1* (11q24), *ERG* (21q22), *ETV1* (7p22), *ETV4* (17q21), or *FEV* (2q33). Because these translocations are not specific to clinical features, fusions of different *ETS* genes to *EWS* can thus be present in phenotypically similar tumors (Delattre *et al*., 1992; Jeon *et al*., 1995; Kaneko *et al*., 1996; Peter *et al*., 1997; Zucman *et al*., 1993b). The *EWS* gene is also involved in several other translocations, leading to the fusion of different DNA-binding proteins to *EWS*, and thus generating distinct tumor phenotypes (Table 1).

In certain soft tissue sarcoma types, ring chromosomes are a frequent finding, sometimes as part of a complex karyotype, but they may also be seen as recurrent aberrations in noncomplex karyotypes, suggesting their pathogenetic role. As sole aberrations or as part of noncomplex karyotypes, ring chromosomes have been reported in MFH, atypical lipoma, well-differentiated liposarcoma, and DFSP, and recently in a case of LGFMS, all of these being low-grade types of soft tissue sarcoma (Bridge *et al*., 1990; Fletcher *et al*., 1996; Heim *et al*., 1988; Mandahl *et al*., 1990; Mezzelani *et al*., 2000; Rosai *et al*., 1996; Örndal *et al*., 1992). Ring chromosomes are mitotically unstable. They show both inter- and intracellular variation in structure and number, thus providing an effective means for gene amplification, yet in other types of malignancies they appear to be rare (Gisselsson *et al*., 1999).

In DFSP, the rings contain material from chromosomes 17 and 22, and low-level amplification of segments from 17q and 22q has been demonstrated in association with the rings (Minoletti *et al*., 1995; Pedeutour *et al*., 1994; Pedeutour *et al*., 1995). The rings have been shown to carry a specific translocation t(17;22)(q22;q13) which results in the fusion of the collagen type I alpha 1 (*COLIA1*) gene with the platelet-derived

growth factor B-chain (*PDGFB*) gene, releasing the latter from its normal regulation (Simon *et al*., 1997) (Figure 2).

In addition to the characteristic translocations and ring chromosomes, other recurrent aberrations, such as deletions, chromosomal gains, double minutes, and homogeneously staining regions, have also been described (Heim & Mitelman, 1995; Sandberg, 1990). Similar to specific translocations, these other cytogenetic findings may also reflect molecular genetic events with important pathogenetic roles, such as tumor suppressor gene inactivation or oncogene amplification described below.

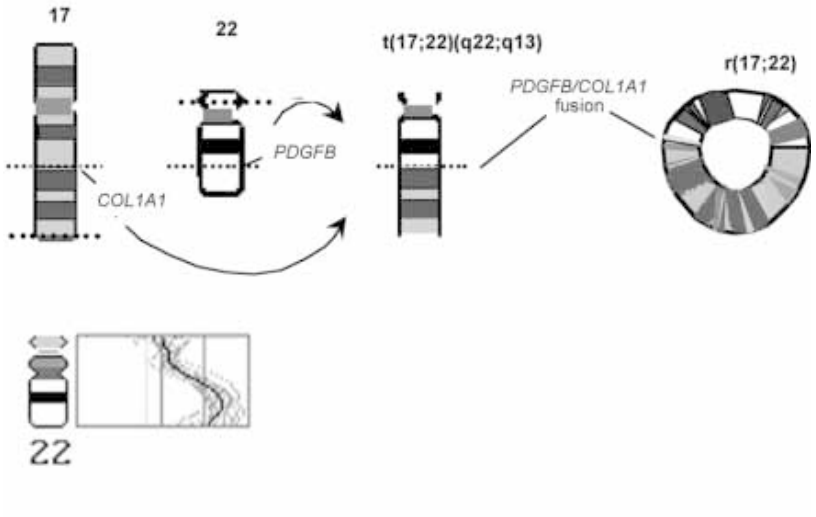

Figure 2. Schematic presentation of the formation of a ring chromosome (17;22) containing amplified segments of both chromosomes and the *PDGFB*/*COL1A1* fusion gene. Below is the CGH profile for chromosome 22 from a dermatofibrosarcoma protuberans tumor (Study IV, case 15) showing high-level amplification of chromosome 22.

Molecular genetic changes

Genes involved in tumorigenesis and tumor progression can be divided into those directly controlling cellular proliferation, e.g., oncogenes and gatekeeper tumor suppressor genes, and those controlling the rate of mutation, called caretaker tumor suppressor genes.

Oncogenes

Oncogenes are altered forms of normal cellular proto-oncogenes. An alteration in a proto-oncogene causes constitutive activation or increased expression of the oncogene, which leads to deregulated cell growth and proliferation. This activation may be caused by a chromosomal translocation, a point mutation, an oncogenic virus, or by gene amplification. Because oncogenes are dominant transforming genes, alteration of only one of the two alleles is sufficient for their tumorigenic potential (Vogelstein & Kinzler, 1998). Constant mutations in oncogenes in soft tissue sarcomas appear to be rare, although mutations in the *RAS* gene family have been suggested to be involved in the development of embryonal rhabdomyosarcoma (Stratton *et al*., 1989a). In gastrointestinal stromal tumor (GIST), mutations of one growth-factor receptor, *KIT*, appear to be recurrent (Hirota *et al*., 1998; Lux *et al*., 2000). These mutations lead to aberrant tyrosine kinase activity. Based on the functional mechanism of the newly synthesized tyrosine kinase inhibitor STI571, first applied in the treatment of CML, Joensuu et al. applied STI571 in the treatment of GIST and reported recently on its efficacy (Joensuu *et al*., 2001).

In addition to fusion gene formation and mutations, oncogenes may be activated by gene amplification, which is commonly seen in soft tissue sarcomas. Gene amplification results in gene activation by overexpression of one or more genes contained in the amplicon. Amplifications are often apparent cytogenetically, either as double minutes, as ring chromosomes, or as homogeneously staining regions (Alitalo *et al*., 1983; Brodeur *et al*., 1981; Cowell, 1982).

Tumor suppressor genes – gatekeepers

Gatekeeper tumor suppressor genes encode proteins that contribute to growth regulation or to differentiation pathways. Instead of activation, functional repression of both alleles of a tumor suppressor gene is needed for the tumorigenic potential. Thus, tumor suppressor genes act recessively at cellular level. Tumor suppressor inactivation may occur as a consequence of mutations or chromosomal deletions, of which the latter may be large enough to be detected cytogenetically (Kinzler & Vogelstein, 1997).

Knudson's "two-hit hypothesis" proposes that of the two inactivating events necessary, the first one may be either germline (inherited) or somatic, and the second one be always somatic (Knudson, 1971). Inherited aberrations in tumor suppressor genes predispose to the hereditary cancer syndromes discussed below, which, although rare, have been of great value for understanding the pathogenesis of various types of sporadic cancers. Retinoblastoma (*RB1*) and tumor protein 53 (*TP53*) genes, for example, both causing hereditary cancer susceptibility when inherited in a mutated form, have proven to be of importance in the pathogenesis of a variety of sporadic cancers, including soft tissue sarcomas (Mulligan *et al*., 1990; Reissmann *et al*., 1989; Stratton *et al*., 1989b; Toguchida *et al*., 1992; Wunder *et al*., 1991). Many different mechanisms for p53 inactivation appear in the literature. In soft tissue sarcomas, a common p53 inactivation mechanism has been suggested to be the result of amplification of the *MDM2* oncogene, whose protein product binds to and functionally inactivates p53 (Oliner *et al*., 1992).

Caretaker tumor suppressor genes - DNA repair genes

Like gatekeeper genes, DNA repair genes are targeted by inactivating mutations. However, unlike the direct involvement of tumor suppressor genes in growth inhibition, DNA repair genes play a less active role in regulating cell growth. Inactivation of both alleles of a DNA repair gene leads to genetic instability, which indirectly promotes growth by leading to an increased mutation rate (Kinzler & Vogelstein, 1997). Known caretaker genes include nucleotide-excision-repair genes responsible for such conditions as xeroderma pigmentosum, and mismatch-repair genes responsible for hereditary nonpolyposis colon cancer, and probably the *ATM* gene responsible for ataxia telangiectasia (Kinzler & Vogelstein, 1997). Recent evidence has implied that the two breast cancer susceptibility genes known, *BRCA1* and *BRCA2*, also possibly fall into this category of tumor suppressor genes (Chen *et al*., 1998; Milner *et al*., 1997; Sharan *et al*., 1997). Genetic instability in a tumor can be observed as defective alleles in microsatellite markers, a phenomenon called microsatellite instability, which has been reported in occasional studies on various soft tissue sarcomas, but the definitive role of caretaker genes in sarcoma pathogenesis is unclear (Bedi *et al*., 1995; Risinger *et al*., 1995; Suwa *et al*., 1999; Wooster *et al*., 1994).

Inherited predisposition

A number of rare genetic diseases significantly increase the risk for certain malignancies. The target genes in familial cancer syndromes usually represent tumor suppressor genes, either gatekeeper or caretaker types, which are often aberrant in sporadic tumors, as well. As Knudson proposed in his "two-hit hypothesis," in familial cancer cases, the first mutagenic event, or "hit," is present in the germline. The “second hit” usually occurs somatically, inactivating the remaining allele of the susceptibility gene. Since the likelihood of the somatic mutation, i.e., the second hit, is high, the predisposition is clinically inherited as an autosomal dominant trait, although, at cellular level a homozygous status is required. Few of the rare syndromes associated with cancer susceptibility caused by mutations in caretaker genes, e.g., xeroderma pigmentosum, are inherited recessively (Vogelstein & Kinzler, 1998).

Familial syndromes with an increased risk for soft tissue malignancies and other cancers include Li-Fraumeni syndrome and familial adenomatous polyposis, which represent typical cancer syndromes, with a high risk for breast cancer and sarcomas, for example, in the former, and for gastrointestinal cancers and desmoid tumors in the latter (Birch, 1994; Burt *et al*., 1995; Clark *et al*., 1999; Li & Fraumeni, 1982). Neurofibromatosis type 1, Beckwith-Wiedemann syndrome, Costello syndrome, and Werner syndrome mainly show manifestations other than malignancies but carry an increased risk for malignancies which include certain soft tissue tumors (Hope & Mulvihill, 1981; Huson *et al*., 1989; Kerr *et al*., 1998).

5.4. COMPARATIVE GENOMIC HYBRIDIZATION IN SOFT TISSUE SARCOMA RESEARCH

Comparative genomic hybridization (CGH) (schematically presented in Figure 3) is based on the comparative hybridization of tumor and normal DNA, each labeled with different fluorochromes, on normal metaphase chromosomes. Differences in the intensities of the two fluorochromes reveal alterations in the amount of DNA sequence in the tumor genome (Kallioniemi *et al*., 1992). Thus, CGH indicates the genomic areas with loss or gain of DNA in the tumor. Consequently, non-balanced translocations may be seen by CGH as gains and losses of the chromosomal segment involved in the translocation. Balanced translocations, which do not change the DNA copy number,

cannot be detected by CGH. Recurrently noted losses suggest inactivation of a tumor suppressor gene(s) in the lost genomic region, and recurrent gains or amplifications indicate activation of an oncogene or of oncogenes within the amplicon (Kallioniemi *et al*., 1992).

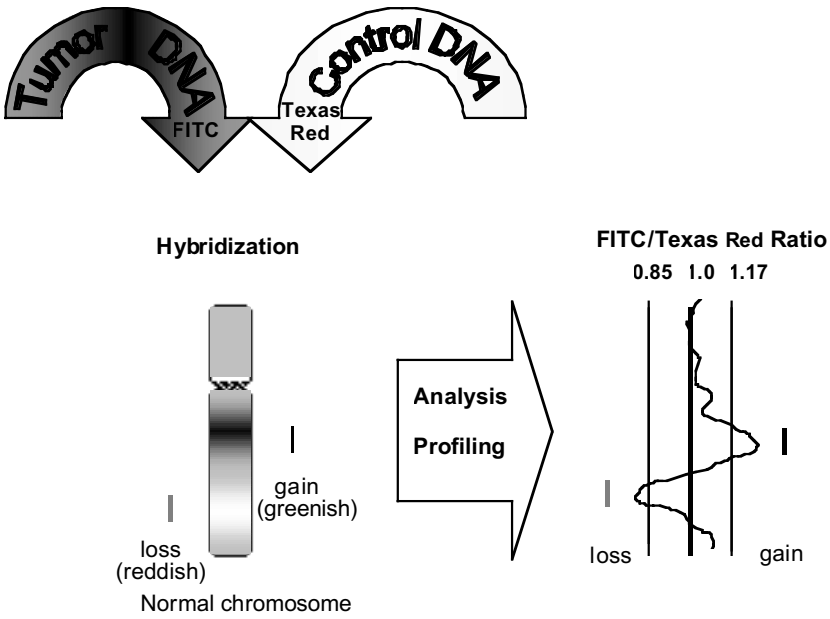

Figure 3. Schematic presentation of the CGH method. Equal amounts of FITC-labeled tumor DNA and Texas Red–labeled normal control DNA are hybridized to normal metaphase chromosomes. The images are evaluated with a special image analysis program. The signal intensities of the different fluorochromes are quantitated along the single chromosomes. The over-and under-represented DNA segments are quantified by computation of FITC/Texas Red ratio images and average ratio profiles.

CGH overcomes the major disadvantages of conventional karyotypic analysis: the requirement of fresh tumor tissue, as well as problems in analyzing complex rearrangements. No mitotic cells are needed, which permits the analysis of tumors with no established cellular proliferation. The main disadvantages of the CGH method include its incapability to discover balanced aberrations, as well as its interpretation problems concerning certain genomic areas, and the requirement of a relatively large amount of either amplified or lost DNA to allow each change to be detected. The genomic areas exhibiting problems in copy number ratio profiling include the telomeric

and heterochromatic regions, which, especially as sole aberrations, cannot be evaluated by CGH reliably (Kallioniemi *et al*., 1994). The sensitivity of CGH is dependent on the proportion of tumor cells versus normal cells in the sample and the level of amplification. An amplicon 1Mb in size may exceed the resolution limit, if it is very highly amplified, and a loss of 10 to 12Mb can be detected, provided the proportion of aberrant cells in the sample is sufficient (Bentz *et al*., 1998; Forozan *et al*., 1997). Recurrent DNA copy number changes have been found useful as diagnostic and prognostic markers for several cancer types, and the CGH method has even been used in localizing genes responsible for inherited cancer syndromes (Hemminki *et al*., 1998; Isola *et al*., 1995; Kainu *et al*., 2000; Moch *et al*., 1996; Suehiro *et al*., 2000). Since soft tissue sarcomas frequently carry extremely complicated chromosomal aberrations, and the tumor tissue is often available only as paraffin-embedded tissue sections, CGH is a highly useful method for analyzing these tumors' genetic aberrations. Consequently, since the introduction of the CGH method in 1992 by Kallioniemi et al., a growing number of CGH studies on different soft tissue tumor types have appeared, comprising today more than 10 different soft tissue sarcoma types and over 700 tumors (El-Rifai *et al*., 2000; Knuutila *et al*., 1999; Knuutila *et al*., 1998; Mezzelani *et al*., 2000; Otano-Joos *et al*., 2000; Pandita *et al*., 1999).

The most frequent copy number changes seen in soft tissue sarcomas include gain of 1q21-q31 which is at least partly present in approximately 25% of the cases analyzed by CGH, followed by gains of 8q and 12q13-q15, at least partly present in approximately 23% and 20%, respectively. Losses are much rarer than gains; the most frequent loss by far is seen at 13q21, present in 22% of soft tissue sarcomas. All of these recurrent changes have been reported in most soft tissue sarcoma types studied by CGH. Gain or high-level amplification of 1q21-q31 is most frequent in LMS (approximately 50%) and liposarcoma (40%); gain or high-level amplification of 8q in embryonal rhabdomyosarcoma (64%); and gain or high-level amplification of 12q13-q15 in both embryonal and alveolar rhabdomyosarcoma (52% and 55%) as well as liposarcoma (47%). Loss of 13q21 is most frequently seen in LMS, being present in approximately 53% of cases. The frequencies have been calculated from nearly 70 original articles, most of them reviewed by Knuutila et al. (El-Rifai *et al*., 2000; Knuutila *et al*., 1999; Knuutila *et al*., 1998; Mezzelani *et al*., 2000; Otano-Joos *et al*., 2000; Pandita *et al*., 1999). The reviews are also available at http://www.helsinki.fi/~lgl_www/CMG.html.

Several recurrent DNA copy number changes have been shown to be of clinical relevance in soft tissue tumor evaluation. In the differential diagnosis of lipoma from liposarcoma, for example, the over-representation of DNA copy number gains at chromosomes 12q and 1q in liposarcomas but not in lipomas can be extremely helpful, especially concerning large and deep-seated lipomas and lipoma-like liposarcomas (Szymanska *et al*., 1997). Patterns of DNA copy number changes which differ between benign and malignant GISTs have, for their part, proven useful in the context of diagnostic and prognostic categorization. Significant differences in changes in several specific chromosomal areas as well as differences in the total number of changes have been detected between benign GISTs, malignant primary GISTs, and metastases (El-Rifai *et al*., 2000; El-Rifai *et al*., 1998a; Sarlomo-Rikala *et al*., 1998). CGH may also be relevant in predicting the prognosis of MFH, since gain of 7q32 is significantly correlated witha worse outcome for the patient(Larramendy *et al*., 1997).

Some of the recurrent DNA copy number gains, including gains of 12q, 11q13, and 17p, have been further studied by molecular genetic methods and been shown to be of complex structure. The 12q13-q15 region, for example, harbors many proto-oncogenes, including *MDM2*, *CDK4*, *SAS*, *CHOP10*/*DDIT3*, and *GLI*. In an amplicon, separate regions, e.g., the one carrying the *MDM2* gene in one segment, and the one carrying the *CDK4* and *SAS* genes in another segment, are co-amplified, whereas the intervening sequences show none or reduced amplification, and even losses of DNA sequences are apparent (Berner *et al*., 1996; Gaudray *et al*., 1992; Wolf *et al*., 1997a; Wolf *et al*., 1997b; Wolf *et al*., 1999). In most instances, however, the target genes and their exact roles in tumorigenesis and tumor progression are yet to be revealed. Since by CGH only a somewhat approximate evaluation of copy number changes is possible, CGH serves mainly as a tool for identifying chromosomal areas of interest to allow further studies by other methods. Recent applications of array technology have enabled the characterization of either copy number changes or gene expression profiling of tens of thousands of single genes or sequences in a single experiment (methods reviewed in *Nature Genetics* supplement issue for vol. 21, 1999). The identification of genetic mechanisms underlying malignant processes will ultimately lead to development of new specific drugs, as already used for CML, and preliminarily also for GIST (Druker *et al*., 2001a; Druker *et al*., 2001b; Joensuu *et al*., 2001).

6. AIMS OF THE STUDY

This study was directed at characterizing genetic alterations in rare soft tissue sarcomas and Ewing tumors. The specific aims were:

To identify DNA copy number changes in alveolar soft part sarcoma, low-grade fibromyxoid sarcoma, Kaposi's sarcoma, dermatofibrosarcoma protuberans, and Ewing tumors, the first three representing tumors with no previously characterized recurrent DNA copy number alterations.

If any recurrent aberrations of special interest were found, to study these further with other methods.

By comparing the copy number changes between typical and fibrosarcomatously transformed dermatofibrosarcoma protuberans, to study whether certain DNA copy number alterations are associated with tumor progression.

To study whether recurrent DNA copy number changes in Ewing tumors have prognostic value.

7. MATERIALS AND METHODS

7.1. MATERIALS

For Study I, 14 ASPS tumor samples from 13 patients were obtained from the Armed Forces Institute of Pathology (AFIP), Washington, DC. The diagnoses were made histologically and further confirmed by immunohistochemical evaluation. The patients were from 9 to 67 years of age (average 25 years), six of them females and eight males. The clinical characteristics of the patients are shown in Table 2.

Study II comprised 11 cases of histologically and immunohistochemically evaluated LGFMS tumors from the Soft Tissue Registry of AFIP. These tumors had occurred in six men and five women, average age 43. Nine were primary tumors in the extremities, and two were pulmonary metastases. The clinical data are shown in Table 2.

Table 2. Clinical characteristics of ASPS and LGFMS patients

Case	Age/ Sex	Tumor Location	Tumor Size
Alveolar soft part sarcoma			
I-1	9/M	Abdominal wall, subcutis	5 cm
I-2	34/M	Scalp	3 cm
I-3	26/F	Thigh	unknown
I-4	23/F	Thigh	10.5 cm
I-5a[1]	26/M	Thigh, deep	6 cm
I-5b[1]	"	"	"
I-6	19/M	Thigh	4.5 cm
I-7	17/F	Upper arm	3 cm
I-8	40/M	Back, intramuscular	5 cm
I-9	18/M	Scalp, subcutis	unknown
I-10	15/F	Shoulder	2.5 cm
I-11	67/M	Spermatic cord	unknown
I-12	9/F	Thigh	2.3 cm
I-13	28/F	Paravaginal tissue	6 cm
Low-grade fibromyxoid sarcoma			
II-1	42/M	Leg	“large”, > 10 cm
II-2	79/F	Buttock	“large”, > 10 cm
II-3	23/M	Leg	14 x 7 x 5 cm
II-4	52/M	Brachial plexus region	11 x 8 x 5 cm
II-5	26/F	Thigh	10.5 x 8.5 x 4.5 cm
II-6	50/M	Upper arm	5 x 4.5 x 2 cm
II-7	54/F	Inguinal region	5 x 3.5 x 3 cm
II-8	27/F	Forearm	5 x 3.5 x 2.5 cm
II-9	29/M	Thigh	5 x 3.5 x 2.5 cm
II-10	50/F	Pulmonary metastasis	variable sizes
II-11	39/M	Pulmonary metastasis	Variable sizes, from 2.5 to 5 cm

[1] 5a and 5b represent two different samples from the same tumor

Study III included histologically and immunohistochemically diagnosed Kaposi's sarcoma samples from 12 patients. Nine of these patients were treated at Helsinki University Central Hospital, Departments of Dermatology (four patients), Surgery (two), Otology (two), and Medicine (one). Seven of the Finnish patients were males; two of them had HIV-infections. Both female patients were HIV-negative, one of them was a Somalian immigrant diagnosed with lupus erythematosus disseminatus (LED) - associated nephropathy. Three samples were obtained from the Soft Tissue Registry of AFIP, from patients who were HIV-positive American males. Clinical data for all Kaposi's sarcoma patients are shown in Table 3.

Table 3. Clinical data for Kaposi's sarcoma patients

Case	Age/Sex	Site[1]	Additional diagnosis[2]	Ethnic origin
III-1	29/F	Skin, arm	LED	Somalian
III-2	29/M	Gingiva	HIV	Finnish
III-3	69/M	Skin, ankle	-	Finnish
III-4	33/M	Subcutis, neck	HIV	Finnish
III-5	73/F	Skin, chin	-	Finnish
III-6	32/M	Scrotum	AIDS	American
III-7	50/M	Tongue	AIDS	Finnish
III-8	35/M	Jejunum	AIDS	American
III-9	77/M	Skin, leg	-	Finnish
III-10	76/M	Skin, foot	-	Finnish
III-11	70/F	Skin, leg	-	Finnish
III-12	61/M	Skin, toe	AIDS	American

[1] site of tumor from which samples were taken (most patients presented with multifocal disease)
[2] LED, lupus erythematosus disseminatus; HIV, human immunodeficiency virus infection

For Study IV, 21 samples from 19 DFSP tumors were obtained from the Soft Tissue Registry of AFIP. Nine of the tumors were classical DFSPs, and ten presented with fibrosarcomatous changes. From two FS-DFSP cases, both typical DFSP areas as well as fibrosarcomatous areas were included separately. The diagnosis was made by histological and immunohistochemical procedures. Clinical data for the patients are shown in Table 4.

Study V included 28 Ewing tumor samples: 16 samples were from Helsinki University Central Hospital, five from the Rizzoli Institute, Bologna, Italy, and five from the Scandinavian Sarcoma Group; 11 cases had been published previously (Armengol *et al*., 1997). Of these tumors, 24 were typical Ewing's sarcomas, one was atypical, and three were primitive neuroectodermal tumors; 16 were soft tissue tumors, and 12 were

bone tumors. All samples were obtained from primary tumors prior to any treatment. Clinical data are shown in Table 5.

Table 4. Clinical data for DFSP patients

Case	Age/Sex	Site	Tumor size	DFSP/ FS-DFSP
IV 1a	52/F	Vulvar area	5.0 cm	DFSP
IV 1b	"	"	"	FS-DFSP
IV 2a	45/M	Abdominal wall	15.0 cm	DFSP
IV 2b	"	"	"	FS-DFSP
IV 3	45/M	Back	6.5 cm	DFSP
IV 4	14/M	Toe	unknown	DFSP
IV 5	36/M	Deltoid muscle	1.3 cm	DFSP
IV 6	65/M	Supraclavic area	7 cm	DFSP
IV 7	47/M	Groin	4.0 cm	DFSP
IV 8	55/M	Chest wall	1.5 cm	DFSP
IV 9	39/M	Toe	1.6 cm	DFSP
IV 10	16/M	Back	unknown	DFSP
IV 11	49/M	Back	unknown	DFSP
IV 12	24/F	Ant. chest wall	unknown	FS-DFSP
IV 13	20/F	Suprascapular area	unknown	FS-DFSP
IV 14	59/M	Buttock	9.0 cm	FS-DFSP
IV 15	42/M	Chest wall	4.1 cm	FS-DFSP
IV 16	36/M	Shoulder	4.0 cm	FS-DFSP
IV 17	37/F	Scapula	2.7 cm	FS-DFSP
IV 18	41/F	Inguinal region	unknown	FS-DFSP
IV 19	72/F	Thigh	9.0 cm	FS-DFSP

Table 5. Clinical data for Ewing tumor patients

Case	Age/Sex	Site/ bone vs. soft tissue	Tumor size	Stage at dg[3]
V-1	24/F	Knee region/soft tissue	18x7x6 cm	M1
V-2	33/M	Calf/soft tissue	10x20 cm	M1
V-3	18/F	Shoulder blade region/soft tissue	9x11x3 cm	M0
V-4	27/F	Femur/bone	10x15 cm	M0
V-5[1]	36/F	Ankle/soft tissue	10x7x3 cm	M0
V-6	19/F	Pelvis/bone	7x5 cm	M0
V-7	33/F	Subcutis, thigh/soft tissue	7x3 cm	M0
V-8	36/F	Buttock/soft tissue	15x8x10 cm	M0
V-9	12/F	Rib/bone	4.4x4.4 cm	M0
V-10	2/M	Ulna/bone	6.5 cm	M0
V-11	26/F	Rib/bone	6 cm	M0
V-12	18/M	Thigh/soft tissue	20x10x5 cm	M0
V-13	21/F	Forearm/soft tissue	8x7x6 cm	M1
V-14	30/M	Calf/soft tissue	unknown	M0
V-15	49/F	Thigh/soft tissue	10x8x5 cm	M1
V-16[2]	26/F	Back/soft tissue	20x10x7 cm	M0
V-17	17/M	Calf/soft tissue	4x3 cm	M0
V-18	20/F	Knee region/soft tissue	8x12 cm	M1
V-19	29/F	Retroperitoneum/soft tissue	7 cm	M0
V-20	28/M	Tibia/bone	6x4 cm	M1
V-21	8/M	Femur/bone	6x6 cm	M0
V-22	32/F	Spine/soft tissue	6x3 cm	M1
V-23	8/F	Femur/bone	10x8 cm	M0
V-24	27/F	Sacrum/bone	9x6 cm	M1
V-25[2]	28/M	Hand/soft tissue	1.5 cm	M0
V-26[2]	18/M	Tibia/bone	15x6 cm	M0
V-27	24/F	Sacrum/bone	10x12 cm	M0
V-28	2/F	Radius/bone	3.5x9 cm	M0

[1] atypical Ewing's sarcoma; [2]primitive neuroectodermal tumor; [3]MO=no metastases at diagnosis, M1= metastases at diagnosis

7.2. METHODS

Comparative genomic hybridization (Studies I-V)

Representative areas of the purest available tumor tissue were chosen for the studies. DNA was extracted from paraffin sections by a salting-out procedure (Miller *et al*., 1988). The comparative genomic hybridization analyses in Studies I to IV were performed as described in El-Rifai *et al*., 1997, and those in Study V as described in Armengol *et al*., 1997. Briefly, in Studies I to IV, tumor DNA was labeled by nick-translation with a mixture of FITC-dCTP and FITC-dUTP (1:1; DuPont, Boston, MA, USA) and normal reference DNA with a mixture of Texas Red-dCTP and Texas Red-dUTP (1:1; DuPont). In Study V, FITC-12-dUTP (DuPont) and Texas Red-dUTP (DuPont) were used accordingly. The nick-translation reaction was optimized to produce DNA fragments of 600 to 2000 bp in length. Equal amounts (800ng) of labeled tumor and control DNA together with 20µg of unlabeled human Cot-1 DNA (Gibco BRL, Paisley, UK) were hybridized to normal metaphase spreads. After a two- to three-day hybridization, the slides were washed, then counterstained with DAPI (Sigma, St Louis, MO, USA), and finally mounted with antifading medium (Vectashield™, Vector Laboratories, Burlingame, CA, USA).

The analysis of the hybridizations was performed with an Olympus fluorescence microscope and the ISIS digital image analysis system (Metasystems GmbH, Altlussheim, Germany). Three-color images of five to nine good-quality metaphases were taken of each sample. The signal intensity ratios of green (FITC) to red (Texas Red) along the chromosomes were calculated. Chromosomal regions were interpreted as over-represented when the ratio exceeded 1.17 (gains), and under-represented when the ratio fell below 0.85 (losses). A ratio over 1.5 served as the cut-off value for high-level amplifications. Results were expressed with a 99% confidence interval.

Polymerase chain reaction (Study III)

HHV-8-specific PCR was used to establish the presence of the virus DNA in six Kaposi's sarcoma samples. The forward and reverse primers 5'-ggagggcacgctagcttcagtg and 5'-tcctcactccaatcccaatgc, respectively, were used to amplify the K12 (or kaposin) open reading frame of HHV-8. Ten epitheloid sarcoma samples,

one DFSP sample, and water served as controls. The PCR cycles were: 94°C for 10 min to activate the enzyme, then 30 times at 94°C for 15 sec, 55°C for 30 sec, and 72°C for 40 sec, then 72°C for 5 min in a reaction mixture containing 200 µM of each dNTP, 0.6 µM of each primer, and 2.5 units of Ampli Taq Gold polymerase in the buffer supplied by the manufacturer (Perkin Elmer Applied Biosystems, Foster City, CA, USA).

The amplified products were separated by gel electrophoresis, transferred to nylon membrane, and hybridized to a digoxigenin-labeled probe. The probe was made by incorporation of DIG-11-dUTP (Boehringer-Mannheim, Mannheim, Germany) into the synthesized DNA strands by PCR, using the same primers and conditions as above.

Interphase fluorescence *in situ* hybridization (Study III)

Two yeast artificial chromosome (YAC) clones, 55G7 and 214D11, served as probes in study of the amplification of the genes *FGF4* and *INT2* (alias *FGF3*) in Kaposi's sarcoma. Both YAC clones covered the region of the closely located *FGF4* and *INT2* genes on chromosome 11q13 (Courseaux A, 1996). The clones were obtained from the Resource Center/Primary Database of the German Human Genome Project (RZPD, Berlin-Charlottenburg, Germany). YAC 950, which hybridizes to 11p16, was used as a control probe. The biotin-14-dATP-labeled probes were hybridized on nuclei extracted from paraffin sections and visualized with FITC. The probes, when hybridized on normal metaphase chromosomes, gave specific hybridization signals at the expected locations.

The nuclei preparations were deparaffinized at 65°C for 15 minutes, followed by xylene treatment (3 x 5 min) at room temperature, and then dehydrated in a descending alcohol series. Approximately 1 µg of the probes was labeled with biotin-14-dATP, and precipitated together with 50 µg of Cot-1 DNA (Gibco BRL), 50 µg of herring sperm DNA (Boehringer Mannheim), 1/10 volume of 3 M sodium acetate, and 14 volumes of absolute ethanol.

Before hybridization, the slides were dipped into 1M sodium thiocyanate at 70°C for 15 min, 0.05 N HCl at 37°C for 10 min, and 0.05 N HCl containing 5 mg/ml pepsin at 37°C for 20 min. The slides were denatured at 75°C for 5 minutes according to standard methods. The hybridization was performed in a moist chamber at 37°C for two to three days.

After hybridization, the slides were washed once in 2 x SSC and twice in 0.1 x SSC at 45°C, for 5 minutes each. The biotinylated probes were detected by avidin-TRITC (1:500, Vector) that was amplified with biotinylated anti-avidin (1:100, Vector) and avidin-TRITC (1:500) with incubations of 40, 45, and 40 minutes, respectively, at 37°C. The counterstaining was performed with 4',6-diamindino-2-phenylindole (DAPI, 2 μg/ml) for 10 minutes.

All probes showed two signals in over 73% of the cells when hybridized on nuclei extracted from paraffin-embedded reactive lymphatic tissue, which served as a control target.

Immunostaining with anti-FGF4 and INT2 antibodies (Study III)

Nine Kaposi's sarcoma tumors were subjected to immunostaining to study whether the amplified *FGF4* and *INT2* genes were expressed in these tumors. Monoclonal anti-human FGF-4 (Sigma) and anti-mouse INT-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as antibodies at dilutions of 1:1000 and 1:50, respectively. Formalin-fixed and paraffin-embedded tumor sections were stained by the avidin-biotin complex immunoperoxidase method with an Elite ABC kit (Vectain, Vector). In each section, non-neoplastic tissue around the tumor tissue served as a control.

Statistical analyses (Studies IV and V)

In Study IV, the total number of CGH changes and the number of 1p34-pter were tested for differences between the DFSP group and the FS-DFSP group by the t-test and Fisher Exact Probability Test, respectively.

In Study V, the aberrations seen in at least three Ewing tumors by CGH (gain or high-level amplification of chromosomes 8, 1q21-q22, 7q, 6p21.1-pter, and 12; and loss of chromosome 16q) and the total number of aberrations per tumor were tested for prognostic significance. Distant disease-free survival (DDFS) and overall survival rates were tested by the Kaplan-Meier method. Associations of copy number changes, tumor size (largest diameter over 8 cm versus 8 cm or less), and tumor location (central versus extremities) with overall survival, DDFS, and local recurrence were tested with the log-

rank test. The statistical significance of the total number of aberrations per tumor was calculated with Cox regression analysis.

8. RESULTS

8.1. COPY NUMBER ALTERATIONS IN ALVEOLAR SOFT PART SARCOMA, LOW-GRADE FIBROMYXOID SARCOMA, KAPOSI'S SARCOMA, DERMATOFIBRO-SARCOMA PROTUBERANS, AND EWING TUMORS (STUDIES I-V)

The CGH results are summarized in Table 6 and Figure 4. Of 86 samples, 84 (81/83 tumors) could be successfully studied by CGH; hybridizations of two of the 12 Kaposi's sarcomas were repeatedly unsuccessful. Of these samples, 61 (58/81 tumors) showed DNA copy number changes (73%). Gains were much more common than losses; 155 single gains were detected, of which 30 were at least partly of high level, compared to only 54 single losses. The most frequent gains or high-level amplifications were those of chromosomes 17q21-qter (24 samples), 22pter-q13 (18), 8q (18, of which 13 were from soft tissue tumors), and 1q21-q22 (13, of which 10 were from soft tissue tumors). The most frequent loss was that of 13q21-q22, seen in six cases. Each of the chromosomes (except for the Y chromosome, which is excluded from the CGH analysis) was affected at least once.

Table 6. DNA copy number changes in ASPS, LGFMS, Kaposi's sarcoma, typical DFSP, FS-DFSP, and Ewing tumors

Case	DNA copy number changes[1]
I	**ASPS**
I-2	+1q, +12q, -18
I-5a	-1p13-p31, -3, +8q, -9p13-pter, +16p, +18p11.2
I-5b	-1p, -3, +8q, +16p,-16q
I-9	+Xp11-pter, -12p, +12q, -21
I-11	+1q, +8q, +16
II	**LGFMS**
II-1	+1q21-q31, +4cen-q22, -4q22-qter, -5q13-q33, -7p, -9p21-pter, -13, -18p, +20q
II-2	-3p13-pter, -3q24-qter, -9p13-pter, -13q14-qter, -14q21-pter, +17q, -21q21-pter
II-3	-6q, -13
II-4	-1q41-qter, -3p12-p22, -13q22-pter, -22q12-qter
II-5	+X, -13, +16p
II-6	+20q11-qter
II-7	-2q34-pter, -4, -7q21-qter, -13q21-qter, +19
II-10	+7q22-q35, +8
II-11	+7q
III	**Kaposi's sarcoma**
III-1	+11q13
III-2	+11q13
III-3	+11q13, +22
III-4	+11q13, +16p
III-5	-6q24-qter, +8q, -10, +16p

Table 6. continued

IV	DFSP - typical
IV-1a	+5, +8, +11, +12p12-pter, +12q24.1-qter, ++17q21-qter, +20, ++22
IV-2a	+17cen-q21, ++17q21-qter
IV-3	+5, -8p12-pter, +17cen-q21, ++17q21-qter, +21q21-qter, +22pter-q13
IV-4	+1, +7, +17q21-qter, +22pter-q13
IV-5	-3q13-q26, +4, +12, -14q21-q32, ++17q21-qter, ++18, +22
IV-6	+5cen-q33, ++17q21-qter
IV-7	+17q21-qter, +22
IV-8	-4p14-pter, ++17q21-qter, +22
IV-9	-6q14-qter, +17cen-q21, ++17q21-qter, ++22pter-q13, +22q13-qter
IV-10	+1q21-q24, +17pter-q21, ++17q21-qter, ++22pter-q13, +22q13-qter
IV-11	+17cen-q21, ++17q21-qter, +22pter-q13
	DFSP with fibrosarcomatous change
IV-1b	++5, +8, +11, +12p, +12q23-qter, +17pter-q21, ++17q21-qter, +20p, ++20q, ++21, ++22
IV-2b	-6q16-qter, +17cen-q21, ++17q21-qter, +22
IV-12	-X, +1, +5q12-q14, +5q23-q34, +6p21.1-pter, +15q21-q25, +17cen-q21, ++17q21-qter
IV-13	-X, ++17q21-qter, +22pter-q13
IV-14	+17q21-qter
IV-15	+1p34-pter, -3cen-q26, +5, +8, -9pter-q22, +9q34-qter, ++17q21-qter, +20q13-qter, +21, ++22
IV-16	+1,+5p13-qter, +16, ++17q21-qter, +18, +21, +22pter-q13
IV-17	+1pter-q31, +7, +12, +13, +17cen-q21, ++17q21-qter
IV-18	+12, +17q21-q22, ++17q22-qter, +22
IV-19	+17pter-q21, ++17q21-qter, +22
V	**Ewing tumors**
V-1	+1q21-22, +8, +12, +14q, +21q
V-4	+4q27-q33, +7q, -11q21-q25, -16
V-5	-3q
V-6	-1p13-p36, +1q21-q31, -9p
V-7	+16q
V-8	+6, +8
V-10	+8
V-11	+4, +8, +12, +14q13-q32
V-12	+7, +8, +16p, +20q
V-13	++6p, +18p, ++19
V-15	+18p11-qter
V-16	+6p21.1-pter
V-17	+7, +8
V-18	+2, +8, +9, +17
V-19	-7
V-20	+1q, -4, +8, -11
V-21	+X
V-22	++1q, -16q, +16p, +17, +22
V-24	+7, +8
V-26	+7
V-27	+1q, +8, +12, -16q

[1] gains of DNA sequences are marked with +, and losses with -. Only cases with aberrations are shown.

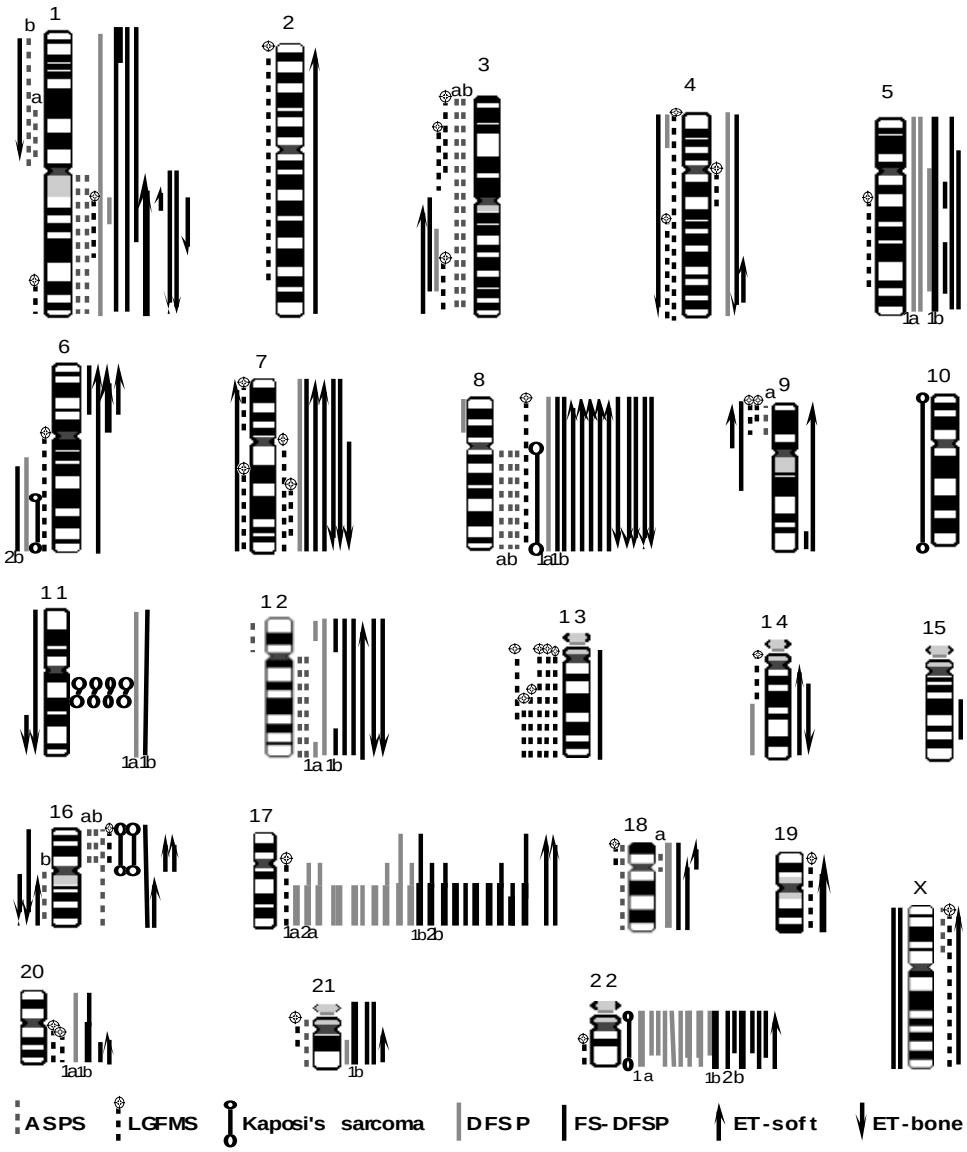

Figure 4. DNA copy number gains and losses for 81 successfully studied tumors. Losses are on the left side of the chromosomes and gains on the right; high-level amplifications as bold lines.
ASPS, alveolar soft part sarcoma (14 samples from 13 tumors); LGFMS, low-grade fibromyxoid sarcoma (11 tumors); Kaposi's sarcoma (10 successfully hybridized tumors); DFSP and FS-DFSP, typical and fibrosarcomatously transformed dermatofibrosarcoma protuberans (11 and 10 samples, respectively, from a total of 19 tumors); ET-soft, Ewing tumor of soft tissue (16 tumors); ET-bone, Ewing tumor of bone (12 tumors).
a and b, different samples from same tumors.

Of these most recurrent changes, gain or high-level amplification of 1q21-q22 was present in four and 8q in all five types of tumors. The 1q21-q22 gain was present in two ASPS cases, five DFSP cases (two of the typical and three of the fibrosarcomatous type), one LGFMS case, and five Ewing tumors (two soft tissue and three bone tumors). Gain of 8q was seen in two ASPS samples (from the same tumor), one LGFMS, one Kaposi's sarcoma, three DFSP samples (two of which represented fibrosarcomatous and typical areas of one single tumor), and ten Ewing tumors (five soft tissue and five bone tumors). The other two gains or high-level amplifications, 17q21-qter and 22pter-q13, were both seen in three different types of tumors: Gain of 17q21-qter was present in one LGFMS, all 19 DFSP tumors (21 samples), and two extraskeletal Ewing tumors. Gain or high-level amplification of 22pter-q13 was present in one Kaposi's sarcoma, 15 DFSP tumors (16 samples), and one extraskeletal Ewing tumor. The most recurrent loss, 13q21-q22, was seen only in LGFMS. The only change in all five types of tumors was gain of 16p, present in two ASPS tumors (three samples), one LGFMS, two Kaposi's sarcomas, one DFSP, and two extraskeletal Ewing tumors.

Proportionally, in distinct tumor types, the most recurrent changes included those already mentioned in DFSP, namely gain or high-level amplification of 17q21-qter, present in 100%, and gain or high-level amplification of 22pter-q13, present in 79% of the DFSP tumors, as well as loss of 13q21-q22 in LGFMS, appearing in 6 of 11 or 55% of the cases.

8.2. HUMAN HERPESVIRUS 8-SPECIFIC SEQUENCES IN KAPOSI'S SARCOMA (STUDY III)

All six tumors from which DNA was available for the PCR study were positive for the K12 (kaposin) open reading frame of HHV-8, yielding an expected 222 bp fragment in gel electrophoresis, whereas all the negative controls were negative.

8.3. THE ROLE OF TWO CANDIDATE GENES AT CHROMOSOME 11q13 IN KAPOSI'S SARCOMA (STUDY III)

Interphase fluorescence *in situ* hybridization and immunohistochemistry

As shown in Table 7, seven of the 12 tumors were studied by interphase fluorescence *in situ* hybridization (FISH) with both YAC probes, 55G7 and 214D11, and nine of the tumors were evaluated immunohistochemically with anti-FGF4 and anti-INT2. One of the hybridizations with 214D11 was unsuccessful. Hybridization of the same tumor with 55G7 gave two signals. This tumor showed weak FGF4 immunoreactivity, and no INT2 reactivity. Two signals with both probes were seen in one case with strong positivity for FGF4 and focal positivity for INT2. All the other tumors gave more than two FISH signals, the mean ranging from three to four signals. These tumors showed variable immunoreactivity; one case was negative for both FGF4 and INT2, two tumors were positive for both, and one tumor was positive for FGF4 but negative for INT2. One of the tumors with more than two FISH signals was not evaluated immunohistochemically because no sample was available. Two tumors available only for immunohistochemistry were both positive for FGF4 and both negative for INT2.

Table 7. Summary of CGH, FISH, immunohistochemical staining, and HHV-8-specific PCR results for 12 Kaposi's sarcoma cases

Case	Copy number changes[1]	Number of FISH-signals[2]		Immunoreactivity of tumor cells		HHV-8 PCR
		YAC 55G7	YAC 214D11	FGF4	INT2	
III-1	+11q13	3(2-5)	3(2-5)	-	-	na
III-2	+11q13	na	na	+	-	+
III-3	+11q13, +22	3(2-5)	3(2-6)	na	na	na
III-4	+11q13, +16p	na	na	+	-	na
III-5	-6q24-qter, +8q, -10, +16p	3(2-5)	3(2-5)	++	++	na
III-6	nr	4(2-5)	4(2-6)	+	+	na
III-7	nr	4(2-6)	3(2-6)	+	-	+
III-8	none	2	2	++	+	+
III-9	none	na	na	++	-	+
III-10	none	na	na	na	na	+
III-11	none	na	na	na	na	na
III-12	none	2	nr	+	-	+

[1] nr, unsuccessful hybridization

[2] na, no sample available

8.4. COMPARISON OF DNA COPY NUMBER CHANGES BETWEEN TYPICAL AND FIBROSARCOMATOUSLY TRANSFORMED DERMATOFIBROSARCOMA PROTUBERANS (STUDY IV)

DNA copy number changes were present in each sample of both the DFSP and FS-DFSP groups. Altogether 47 copy number changes were observed in the 11 DFSP samples (mean 4.3 changes per sample) and 57 changes in the 10 FS-DFSP samples (mean 5.7 per sample). The number of changes ranged from 2 to 8 in the DFSP group, and from 1 to 11 in the FS-DFSP group. High-level amplifications were observed in 13 DFSPs and 14 FS-DFSPs.

The most recurrent change was gain or high-level amplification of 17q21-qter, which was present in all samples in both groups. This was followed by gain or high-level amplification of 22pter-q13, present in nine (82%) and seven (70%) of the DFSP and FS-DFSP samples, respectively. The third most common change was seen at 5q, at which two minimal overlapping areas (5q12-q14 and 5q23-34) were gained or amplified in three DFSP and four FS-DFSP samples (27% and 40%, respectively).

Comparison of the changes in the DFSP and FS-DFSP groups revealed no statistically significant differences: the clearest differences occurred in the total number of changes (P=0.24), and gain of 1p34-pter, which was present in one DFSP sample and in four FS-DFSP samples (P=0.15).

8.5. PROGNOSTIC SIGNIFICANCE OF DNA COPY NUMBER CHANGES IN EWING TUMORS (STUDY V)

The total number of aberrations detected by CGH per tumor showed no statistically significant correlations with the prognostic parameters (P=0.08 for DDFS and 0.13 for overall survival). Among the most frequent aberrations tested for prognostic impact, certain associations were found, none of which was, however, statistically significant: the most frequent aberration, gain of chromosome 8 (present in 36%), was associated with trends towards worse DDFS (P=0.16) and worse overall survival (P=0.39). Gain or high-level amplification of 1q21-q22 (present in 18%) showed similar associations (P=0.30 and 0.45, respectively). The DDFS and overall survival of patients with gain of 7q

(present in 18%) were slightly better than of those without 7q gain (80% versus 51%, *P*=0.30 for DDFS; and 75% vs. 53%, *P*=0.45).

Gain or high-level amplification of chromosome 6p21.1-pter (present in 11% of the tumors) was associated with poor prognosis: none of the patients with this aberration survived for 5 years, whereas the 5-year overall survival of the patients without this aberration was 64% (*P*=0.004). Similarly, the 5-year DDFS among the patients with and without this imbalance was 0% and 63%, respectively (*P*=0.04). Gain of chromosome 12 (present in 11%) was associated with a trend towards worse DDFS (*P*=0.36), but no association was detectable between this gain and overall survival (*P*=0.67). The most frequent loss at 16q (appearing in 11%) showed no correlation with any of the prognostic parameters.

9. DISCUSSION

9.1. EXTRACTING INFORMATION FROM SOFT TISSUE SARCOMAS BY COMPARATIVE GENOMIC HYBRIDIZATION

CGH overcomes many problems associated with conventional cytogenetic analysis. First, the requirement of only DNA instead of viable cells allows the use of archival tumor material, which is especially important when studying rare tumors. Even in the presence of fresh tumor samples, cellular proliferation is often difficult to establish for banding analysis. Second, by CGH the overview of even a very complex karyotype can easily be achieved. Although CGH is an efficient method for screening DNA copy number changes, it cannot, however, be used to analyze amplification of single genes, not to mention gene expression. Thus, once the interesting copy number alterations have been found, the genes of interest must be further studied by other molecular genetic methods. In addition to revealing chromosomal segments carrying genes with potential importance in tumorigenesis and tumor progression, DNA copy number changes may be useful in clinical assessment, such as differential diagnostics, tumor categorization and prognostic evaluation.

In this work, CGH has been applied in the study of archival samples of very rare soft tissue sarcomas and Ewing tumors. No satisfactory number of fresh tumor samples of this nature could have been obtainable for a search for aberrations by conventional methods. DNA copy number changes of the rare ASPS, Kaposi's sarcoma, and LGFMS tumors were reported during these studies for the first time, and new information on copy number changes in DFSP as well as in Ewing tumors were added to those previously described. The frequencies of the most common changes seen in the present study are compared in Table 8 to previously described frequencies in other soft tissue sarcomas.

Table 8. Frequency of recurrent copy number changes in present and previous studies

	Number of tumors with the copy number change (%)[1]				
	Gain or high-level amplification				Loss
	17q21-qter	22pter-q13	8q	1q21-q22	13q21-q22
Studies I-V (69 tumors)[2]	22 (31%)	17 (24%)	11 (15%)	10 (14%)	6 (8%)
Other studies (517 tumors)[3]	62 (12%)	36 (7%)	99 (19%)	106 (21%)	110 (21%)
Malignant fibrous histiocytoma (155 tumors)	15 (10%)	17 (11%)	18 (12%)	32 (21%)	35 (23%)
Leiomyosarcoma (72)	12 (17%)	11 (15%)	21 (29%)	27 (38%)	38 (53%)
Synovial sarcoma (76)	8 (11%)	1 (1%)	10 (13%)	5 (7%)	11 (14%)
Embryonal rhabdomyosarcoma (33)	4 (12%)	2 (6%)	19 (58%)	4 (12%)	1 (3%)
Alveolar rhabdomyosarcoma (55)	9 (16%)	2 (4%)	10 (18%)	13 (24%)	5 (9%)
Liposarcoma (55)	4 (7%)	3 (5%)	11 (20%)	20 (36%)	6 (11%)
Gastrointestinal stromal tumor (71)	10 (14%)	0	10 (14%)	5 (7%)	14 (20%)

[1] Tumors with the change covering more than half of the region mentioned
[2] Comprising 13 alveolar soft tissue sarcomas, 11 low-grade fibromyxoid sarcomas, 10 Kaposi's sarcomas, 19 dermatofibrosarcoma protuberanses, 16 soft tissue Ewing tumors
[3] References: (El-Rifai *et al*., 2000; Knuutila *et al*., 1999; Knuutila *et al*., 1998; Otano-Joos *et al*., 2000; Pandita *et al*., 1999)

9.2. WHAT DO COPY NUMBER CHANGES TELL ABOUT THE TUMORS?

No specific copy number changes in alveolar soft part sarcoma

The molecular cytogenetic background of ASPS was discovered after Study I was published. A specific translocation der(17)t(X;17)(p11;q25) was identified, similar to many sarcoma types (Joyama *et al*., 1999). This rearrangement brings extra chromosomal material from Xp11-pter to chromosome 17q25, and leads to a novel fusion gene between *TFE3* transcription factor at Xp11.2 and a novel gene, *ASPL*, at 17q25 (Ladanyi *et al*., 2001). Interestingly, one of the ASPS tumors in Study I showed gain of Xp with the starting point coinciding with band p11. This fits well with the non-reciprocal nature of the translocation with excess material from Xp in at least some of the cases (Joyama *et al*., 1999). Compared to other specific translocations, typically of the balanced type, one could speculate that the excess chromosomal material introduced by the unbalanced situation might itself carry some pathogenetic importance in addition to the fusion gene formation.

Copy number aberrations present in two ASPS tumors each included gains of 1q, 8q, 12q, and 16p. The first three represent the most frequent aberrations in soft tissue sarcomas in general, and are unspecific as to tumor types. The high prevalence of these changes in different sarcoma subtypes may suggest that the same oncogenes act in the progression of both the more common as well as the very rare sarcoma types. Gain of 16p has most frequently been reported in LMS (approximately 26%). Considering the smooth muscle origin of LMS, it is of interest that for ASPS, skeletal muscle differentiation of the tumor cells has been suggested. The 16p gain has, however, been reported in several other sarcomas as well, and thus cannot be taken as suggestive of either a skeletal or a smooth muscle origin of this tumor (Foschini *et al*., 1988; Knuutila *et al*., 1998; Miettinen & Ekfors, 1990a; Mukai *et al*., 1989; Persson *et al*., 1988).

Loss of 13q21-q22 is especially frequent in low-grade fibromyxoid sarcoma

Losses at 13q, the minimal overlapping area spanning 13q21-22, were seen in six LGFMS tumors (55%), but not in any other of the sarcoma types. Although loss of 13q21 is the most frequently reported copy number loss in soft tissue sarcomas studied previously by CGH, (present in approximately 20% of the cases overall), the highest frequency thus far has been in LMS, which shows this aberration in approximately 53% of cases (Knuutila *et al*., 1999; Otano-Joos *et al*., 2000). Altogether, losses were much more common in LGFMS than gains, which is untypical of soft tissue sarcomas in general. Previously, only one LGFMS case with genetic aberrations had been reported, a tumor showing gains of 7p14-pter, 7q31-q33, and 16p, as well as losses of chromosomes 18 and 21q and no losses of chromosome 13 (Mezzelani *et al*., 2000). The universal phenomenon of 13q losses in various soft tissue sarcomas, including LGFMS, may indicate a common step in the genetic process of several sarcoma types. Then again, this aberration appears to be exceptionally frequent in LGFMS, which may imply an even more LGFMS-specific role for an as-yet-unidentified tumor suppressor gene or genes located at 13q21. All the losses present in at least two LGFMS cases are frequent also in MFH, which shares similarities with LGFMS (Larramendy *et al*., 1997; Mairal *et al*., 1999; Parente *et al*., 1999). Indeed, when discussing the classification of LGFMS in his report of 12 cases, Evans considered the possibility of LGFMS

representing a subtype of MFH, but concluded that they belong to different entities (Evans, 1993).

Gains of 11q13 in Kaposi's sarcoma indicate amplification of oncogenes from this restricted area

Four of the ten successfully hybridized Kaposi's sarcoma tumors (40%) showed gain of 11q13, covering only the q13 band; in two tumors this was the only detectable change. Gain of 11q13 has been reported in several other soft tissue sarcomas, in which it is not, however, among the most frequent. In LMS and MFH, the 11q gain is similarly often restricted to the q13 band (Larramendy *et al*., 1997; Mairal *et al*., 1999; Menghi-Sartorio *et al*., 2001; Otano-Joos *et al*., 2000). In breast cancer, in which the amplification of 11q13 is also frequent, the amplicon contains several cores of amplification, one being centered around the *CCND1* gene, and other cores carrying such genes as *EMS1*, *BCL1*, and *INT2* (Karlseder *et al*., 1994; Schuuring *et al*., 1992). The *INT2* gene as well as the *FGF4* gene, also located at 11q13, belong to the fibroblast growth factor family, whose members have been studied as possible pathogenetic factors in Kaposi's sarcoma (Goldman *et al*., 1997; Murakami-Mori *et al*., 1998). Because of the previously observed link between Kaposi's sarcoma and the FGF genes, as well as the link between the mouse homolog of *INT2* and malignancies caused by a tumor virus, these two genes, located at 11q13, were further studied by fluorescence in situ hybridization and immunohistochemistry.

Consistency of 17q and 22q gains in dermatofibrosarcoma protuberans suggests important roles for these changes

The two most frequent copy number changes seen in our studies (I-V) were gain or high-level amplification of 17q21-qter, seen in 29% of the samples; and gain or high-level amplification of 22pter-q13, seen in 21%. The high frequency of these changes was caused almost exclusively by their high prevalence in DFSP. In addition to DFSP, in which gain or high-level amplification of 17q21-qter was present in 100% of the cases, only two Ewing tumors and one ASPS showed this change. Gain or high-level amplification of 22pter-q13 was present in 79% of the DFSP samples, but in only one Kaposi's sarcoma and one Ewing tumor. These findings fit previous cytogenetic findings

in DFSP, which include ring chromosomes shown to carry material from chromosomes 17 and 22. Furthermore, the starting points of the gains or amplifications on chromosome 17 were often located around the locus for *COL1A1*, one of the fusion gene partners. Previously, gains of 17q segments have been reported in 8/12, and gains of 22q segments in 10/12 DFSP tumors studied by CGH (Naeem *et al*., 1995; Pedeutour *et al*., 1995).

In other soft tissue sarcomas, gain or high-level amplification of 17q appears most frequently in LMS and alveolar rhabdomyosarcoma, being seen in approximately 20% of these tumors, although other more recurrent changes are typically seen in both tumors. In MPNSTs of patients with neurofibromatosis type 1, 17q gain has also been reported to represent a characteristic change. Chromosome 22 gain is more rare, most often described in LMS (approximately 12%) (El-Rifai *et al*., 1998b; Forus *et al*., 1995; Gordon *et al*., 2000; Levy *et al*., 2000; Otano-Joos *et al*., 2000; Packenham *et al*., 1997; Pandita *et al*., 1999; Parente *et al*., 1999; Schmidt *et al*., 2000; Tarkkanen *et al*., 1999b; Weber-Hall *et al*., 1996).

The high prevalence of 17q and 22q amplifications strongly suggests that the acquisition of material from these genomic segments has an important impact on the pathogenesis of DFSP, independent from or associated with the translocation. A finding suggesting an analogous situation has been reported in alveolar rhabdomyosarcoma, in which the PAX3-FKHR fusion gene, generated by a specific translocation, was insufficient for tumor formation in knock-out mice. The authors speculated that additional genetic events are needed for development of sarcomas (Xie *et al*., 2001). Amplification of distinct genes from the 17q21-qter area has been described in several malignancies, including the genes *PAT1*, *PS6K*, *SIGMA1B*, *TBX2*, and *RAD51C* in breast cancer, *PS6K* in anaplastic meningioma, and *GAS* in gastric cancer. The definitive role of these in the carcinogenesis is, however, still unclear (Bärlund *et al*., 2000; Cai *et al*., 2001; Vidgren *et al*., 1999; Wu *et al*., 2000).

Similar changes in typical and fibrosarcomatously transformed dermatofibrosarcoma protuberans indicate mechanisms other than copy number changes as acting in tumor progression

Differences in copy number changes between the classical DFSPs and the FS-DFSPs were evaluated to study possible copy number alterations in tumor progression.

Differences in the most common changes--17q and 22 gains--between the two groups were negligible; in fact, the 17q gain was present in every sample in each group. In addition to the independent importance of these gains in DFSP, these findings suggest also that the 17q and 22 gains are among the primary genetic aberrations with an essential role in the early tumorigenetic process, but no substantial role in the progression of DFSP.

The clearest, although not significant, differences between the two groups were noted in the total number of aberrations and in the gain of 1p34-pter. An increasing number of copy number changes have been reported to correlate with tumor progression in several malignancies such as LMS, GIST, cervical cancer, endometrial cancer, and renal cell cancer (Allen *et al*., 2000; El-Rifai *et al*., 2000; El-Rifai *et al*., 1998b; Jiang *et al*., 2000; Junker *et al*., 2000; Suehiro *et al*., 2000). The accumulation of copy number changes during progression fits well with the multi-step model of carcinogenesis. The 1p34-pter area is recurrently amplified in the alveolar subtype of rhabdomyosarcoma, in which the amplicon contains a variant fusion gene partner *PAX7* at 1p36, which (alternatively with *PAX3* at 2q35) is fused to *FKHR* at 13q14 (Barr *et al*., 1996; Gordon *et al*., 2000; Weber-Hall *et al*., 1996). Recurrent gain of 1p34-pter with *PAX7* amplification has also been described in lung carcinoma, but the definite role of the *PAX7* gene or other genes from 1p34-pter in tumor progression has not been established (Racz *et al*., 2000).

Although the total number of aberrations in both the DFSP and the FS-DFSP group was relatively high (mean 4.3 and 5.7 per sample, respectively), no significant differences were noted between the groups. This may imply that the genetic events during progression from DFSP to FS-DFSP include other more important genetic mechanisms undetectable by CGH.

Non-specific gains of 8q and 1q21-q22 may have prognostic value in Ewing tumors

In Studies I to V, gain of 8q and gain or high-level amplification of 1q21-q22 were present in 21% and 15% of all samples. The 8q and 1q21-q22 gains appeared in five and four tumor types, respectively, reflecting the high overall frequency but the lower specificity of these changes in soft tissue sarcomas. Both changes have been reported in almost all

soft tissue sarcoma types, as well as in other types of cancer studied by CGH. Both gains were most frequently seen in the Ewing tumors, in which they were also the most recurring changes. Gain or high-level amplification of 1q21-q22 was present in five (18%), and gain of 8q in ten (36%) of the Ewing tumors. In two cases, loss of 16p was present simultaneously with gain of 1q, which cytogenetically is often seen as a der(1;16).

In the statistical analysis of the associations between copy number changes and clinical parameters, a trend towards worse DDFS and overall survival was associated with 1q21-q22. This association was, however, not statistically significant because of the small number of patients. Previously, gains at 1q21-q25 have been associated with a metastatic phenotype or short overall survival in renal clear cell carcinoma, high-grade osteosarcoma, and prostate cancer (Alers *et al*., 2000; Gronwald *et al*., 1997; Tarkkanen *et al*., 1999a). The 1q21-q22 area carries several genes with potential significance in sarcoma pathogenesis, many of which, e.g., the *FLG*, *NTRK1*, and *SPRR3*, have been shown to be amplified in different types of sarcoma (El-Rifai *et al*., 2000; Forus *et al*., 1998; Knuutila *et al*., 1998; Mezzelani *et al*., 2000; Otano-Joos *et al*., 2000; Pandita *et al*., 1999).

The most common change in the Ewing tumors, gain of chromosome 8, was also associated with a non-significant trend towards worse prognosis. When compared to the prognosis for patients without 8q gain, this change was seen as lower 5-year DDFS (40% vs. 65%) and overall survival (45% vs. 63%). Gains of chromosome 8, or parts of it, have previously been reported to correlate with shorter survival in osteosarcoma, and with larger tumor size in LMS as well as in synovial sarcoma (El-Rifai *et al*., 1998b; Hattinger *et al*., 1996; Maurici *et al*., 1998; Mugneret *et al*., 1988; Skytting *et al*., 1999b; Tarkkanen *et al*., 1999a).

A trend towards worse DDFS was connected also with gain of chromosome 12, present in three Ewing tumors. Chromosome 12 represents one of the most frequently gained segments in sarcomas, the most frequent restricted amplicon spanning 12q13-q15, which harbors several oncogenes known to be amplified in different sarcomas. The exact roles of these genes in tumor progression are, however, as yet unknown (Knuutila *et al*., 1998).

An association of gain or high-level amplification of 6p21.1-pter with poor outcome was noted; patients with and without this change had a 5-year DDFS of 63% and 0%

(P=0.04) and overall survival of 64% and 0% (P=0.004), respectively. One of the three patients with this aberration had metastatic disease at the time of diagnosis, and two had large central tumors, all representing clinical parameters that have been associated with poor prognosis. It is thus difficult with this small patient series to estimate the significance of 6p21.1-pter for prognosis independent of other prognostic markers, since the aberration may be the cause of other prognostic variables, or be merely a by-product in the progression of the disease.

Overall, the fact that relatively few copy number changes were present in the tumors may reflect the significance of the primary genetic event, the translocation, in the pathogenesis. Recently, study of an extended patient series of 134 Ewing tumor patients, including the patients of Study V, could confirm a significant association between gain of 1q and poor prognosis. In addition, gain of chromosome 12 was found to predict poor outcome in patients with localized disease, and loss of 16q was significantly associated with disseminated disease at diagnosis. No associations were established between 6p gain and prognosis (Hattinger *et al*., unpublished).

9.3. *INT2* AND *FGF4* ONCOGENES ARE AMPLIFIED AND EXPRESSED IN HUMAN HERPESVIRUS 8-POSITIVE KAPOSI'S SARCOMA

The HHV-8-specific sequence of the kaposin gene was present in all six samples tested. This novel herpesvirus was first found to be associated with malignancy when identified in a Kaposi's sarcoma skin lesion of an AIDS patient in 1994; it has since been strongly implicated as a causative factor in Kaposi's sarcoma and other malignancies such as primary effusion lymphoma and multicentric Castleman's disease (Chang *et al*., 1994; Dupin *et al*., 1999; Mayama *et al*., 1998; Renwick *et al*., 1998; Schulz, 1998). HHV-8 belongs to the same herpesviridae family as EBV, a human tumor virus associated with Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, and possibly other tumors (Butel, 2000). EBV acts by encoding the oncogene protein LMP1 (latent infection membrane protein 1), which mimics an activated growth factor receptor and interacts with factors that mediate its proliferative signals (Mosialos *et al*., 1995). The mechanistic role of HHV-8 in cancer development is still unclear, but its genome harbors several genes with homology to the cellular genes involved in cell-cycle

regulation, cell proliferation, apoptosis, and immune modulation (Boshoff & Chang, 2001).

The *INT2* gene, located at 11q13, is a homolog of the mouse oncogene *int2*; the *int2* oncogene is activated by a tumor virus, MMTV, which integrates a proviral sequence into the vicinity of the proto-oncogene (Butel, 2000). The *FGF4* gene, in close proximity to *INT2* at 11q13, was first identified in Kaposi's sarcoma cell transfection experiments (Delli Bovi & Basilico, 1987). Because of these associations--viral tumorigenesis for the *INT2* gene and identification of the *FGF4* gene in Kaposi's sarcoma cells--we chose to study the amplification and expression of these genes, and found them to be aberrant in almost all tumors available for these studies. The mean number of FISH signals of the probes which were carrying both genes was 2.8 per tumor, and immunoreactivity of either INT2 or FGF4 was present in 8/9 cases. Amplification of 11q13 has previously been described in several malignancies, including breast cancer, oral squamous cell carcinoma, and melanoma. The amplicon harbors the *INT2* and *FGF4* as well as other genes like *CCND1/PRAD1* and *EMS1*, which have been shown to be amplified in a discontinuous way, the amplification and expression pattern varying between different tumors (Adelaide *et al*., 1988; Hui *et al*., 1997; Lese *et al*., 1995; Shuster *et al*., 2000; Szepetowski *et al*., 1992). Both *INT2* and *FGF4* are normally expressed during embryogenesis. *Int2* has been connected to inner ear development in the mouse, and *fgf4* participates in limb development in vertebrates (Johnson & Tabin, 1997; Represa *et al*., 1991). Thus, the immunoreactivity of either FGF4 or INT2 seen in the Kaposi's sarcoma tumors suggests that expression of these genes, possibly caused by amplification, may play a role in Kaposi's sarcoma pathogenesis. Although the mechanism of tumor induction by promoter insertion--such as MMTV provirus integration leading to *int2* activation and subsequent breast cancer in the mouse--appears uncommon in virus-induced human cancer, the activation of *INT2* in the presence of a novel human tumor virus warrants consideration of similar integration mechanisms, in addition to the signaling cascade mechanisms suggested in transformation (Chow *et al*., 2001; Renne *et al*., 2001; Samaniego *et al*., 1998).

10. CONCLUSIONS AND FUTURE PROSPECTS

This work was aimed at characterizing genetic alterations in rare soft tissue sarcomas, and evaluating the role of these aberrations in tumor progression and disease prognosis in appropriate contexts. CGH proved an efficient screening tool for analyzing archival samples of tumors from which fresh material was unavailable for the more conventional analyzing methods. In this work, copy number alterations of the very rare ASPS, LGFMS, and Kaposi's sarcoma were described for the first time. In addition, new information on copy number changes in Ewing tumors and DFSP has been added to that previously available.

After Study I was published, a specific der(17)t(X;17)(p11;q25) of non-reciprocal nature was reported in ASPS. In one of the tumors in Study I, this translocation was presumably reflected by a gain of Xp11-pter. Eight of the 13 tumors revealed no DNA copy number changes, which may indicate that the translocation in some of the tumors exists in a balanced form. Additional balanced aberrations may also be significant in ASPS pathogenesis.

LGFMS exhibited an exceptionally frequent loss of 13q21-q22. Although this alteration is common in various types of sarcomas, its high prevalence in LGFMS may be indicative of its more specific role in this tumor type. Further studies of this genomic region with no currently known oncogenes may reveal genes with importance in LGFMS as well as many other sarcomas.

In Kaposi's sarcoma, a recurrent gain was found of a restricted chromosomal region, and two oncogenes from this region with special theoretical interest were further investigated by FISH and immunohistochemistry. The studies revealed that the *INT2* and *FGF4* oncogenes were indeed both amplified and expressed in the tumors. These findings are of special interest in the context of Kaposi's sarcoma, its being a malignancy caused or influenced by a virus whose exact tumorigenic mechanisms are still unclear. Further studies will be needed to evaluate the oncogenic roles of the activated human genes *INT2* and *FGF4* and their functional mechanisms in viral tumorigenesis.

The specific translocation of DFSP is often located at ring chromosomes carrying material from the chromosomes involved in the translocation. The finding that gains or amplifications of the translocation chromosomes are almost exclusively present in the

tumors strongly suggests that these regions carry oncogenes with primary importance for tumorigenesis. Based on similarities between the typical and fibrosarcomatously transformed tumors, it can instead be concluded that in the progression process copy number alterations are not important.

In Ewing tumors, copy number gains of 8q and 1q21-q22 were shown to be associated with trends towards worse prognosis. The number of patients was not sufficient, however, for revealing statistically significant associations. In addition to these recurrently described changes in different sarcomas, gain of 6p was associated with poor prognosis. Because this change was seen in tumors with clinical markers of poor prognosis, however, the independent value of this gain was not interpretable. Since new prognostic markers are of great importance when evaluating these highly malignant tumors, further analyses will be needed with larger patient series. When evaluating such a rare malignancy, statistically significant correlations can be achieved only with international collaboration. Such a trial with an extended patient series has now been started, and the preliminary results support the finding of 1q gain predicting poor prognosis for Ewing tumors.

The less specific copy number changes seen in this study are also prevalent in other subtypes of soft tissue sarcomas. The DNA copy number gains and losses detected by CGH are in many cases likely to represent unbalanced chromosomal translocations. Further clarification of the nature of these possibly extremely complex rearrangements can be conducted with the recently developed FISH techniques such as multicolor FISH and spectral karyotyping (Schrock *et al*., 1996; Speicher *et al*., 1996). The chromosomal areas with common aberrations may harbor genes playing important roles in the common pathogenetic pathways of these as well as of other malignancies. In the future, use of novel array techniques may offer an explicit view of both the specific as well as the more common target genes within the amplified or lost regions, and the expression of these genes may be evaluated in detail. Identifying these molecular mechanisms will undoubtedly lead to the development of drugs specifically targeting the genetic defects underlying different sarcomas, as is already the case in at least some of the GISTs.

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